20 YEAR EVOLUTION OF GLYADERM® DERMAL REGENERATION MATRIX

THE FIRST NON-COMMERCIAL DERMAL REGENERATION MATRIX

ALI PIRAYESH

20 year evolution of Glyaderm® dermal regeneration matrix.

The first non-commercial
dermal regeneration matrix.

Ali Pirayesh

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by

Ali Pirayesh

born on 22 November 1969
in Tehran, Iran

Supervisors
Prof. B. van der Lei, PhD
Prof. S. Monstrey, PhD

Assessment committee
Prof. A. Mosahebi, PhD
Prof. B. Horvath, PhD
Dr. A. Piatkowski de Gryzmala, PhD

Paranimfen
Mr. T. Wagemans
Drs. A. Bax

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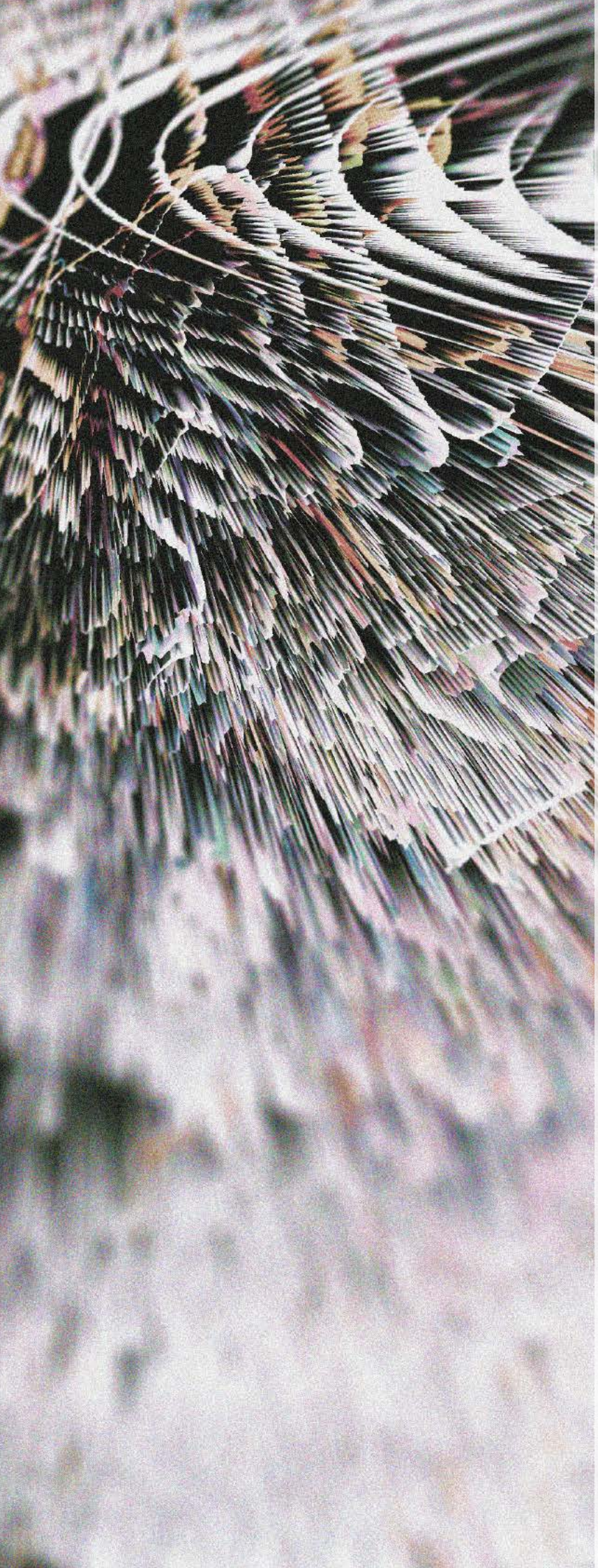

CHAPTER 1

INTRODUCTION

Introduction and outline of this thesis

Introduction

Severe Burns remain a major cause of injury-related mortality [1]. Advancements in burn care and surgical burn reconstruction have led to improved patient survival and rehabilitation. Currently, the primary focus of burn care has shifted from only survival to improving the quality of patient survival [2,3]. The quality of life of burn patients is largely influenced by their ability to re-integrate into society which is associated with scar quality and appearance, and perception of their own appearance.

Split thickness skin graft (STSG) remains the gold standard for surgical reconstruction of deep dermal and full thickness burns [3,4]. However, STSG is associated with challenges such as a paucity of donor sites, donor site morbidity, graft contracture, and/or unfavourable and unpredictable scarring [5,6].

The development of biological, synthetic skin substitutes, and human allografts has augmented the armamentarium of the burn clinician with alternatives to autologous STSG [5].

Dermal regenerative matrices (DRMs) are permanent skin substitutes that allow for a degree of regeneration of the dermal skin component in the management of major burns, traumatic contractures, and skin defects [7]. The use of a DRM in burn surgery has shown to produce a more favourable functional and aesthetic results [8–12].

Drawbacks for the use of DRM include the need for a two-stage procedure [13–15], increased infection risk [13–15], and high cost [12,15].

Historical aspects and aim of our studies

Historical Aspects

At the start of the millennium our research group was faced with challenges in burn care reconstruction. The DRM Integra was gaining attention in literature and at conferences but the variability in take rate and high cost prevented its widespread application and routine use in the Gent Burn Unit.

Pirayesh who had witnessed the savage burn trauma in revolution and war as a child in Iran was grasped by Plastic Surgery & Burn Care as a Senior House Officer at the burn unit in East Grinstead where his mentor Philip Gilbert taught him the principles of burn care passed on by Sir Archibald McIndoe who had bravely treated the burns of RAF pilots from the Battle for Britain. He started research into keratinocyte culture and presented papers at burn conferences where he met Hans Hoekstra, the inventor of the glycerol preserved allograft (GPA). Hoekstra was active in experimental burn research in Amsterdam and taught Pirayesh the core principles of experimental burn research together with Dr Nelleke Richters who worked as an immunologist and researcher for the Dutch Burn Foundation.

Pirayesh was impressed by the research output from the Gent Plastic Surgical Unit and approached Prof Stan Monstrey at a conference who gave him the opportunity to

apply for a residency. Pirayesh was selected for residency and had to start with a pre-residency year at the Gent Burns Unit. Henk Hoeksema, the principle burn care coordinator taught him the principles of conservative burn care and surgical burn care. They introduced and initiated studies with MEEK grafting and interactive honey dressings in the Gent unit which was famous for the use of laser doppler imaging to scientifically delineate burn depth and therefore the ideal place for clinical studies on burns.

Their collaborative brainstorming sessions culminated in the idea of developing a dermal substitute based on glycerol preserved allograft which would be a non-profit and cost-effective DRM for widespread application and improvement of the quality of life of the burn patients.

Pirayesh, Hoeksema, Richters, Hoekstra and Monstrey signed away their IP rights for the DRM "Glyaderm" which was affirmed by notary to the EuroSkinBank, now EuroTissueBank, Beverwijk, The Netherlands.

Pirayesh returned to the Netherlands to build his private practice, but propagated Glyaderm® research and global application which resulted in the development of a Colombian Glyaderm® for victims of acid attacks. The Two Faces *(https://twofacesfoundation.org)* charity was set up to help these victims by his partner Eva Velders.

Berend van der Lei, together with Prof Monstrey, have been inspiring forces throughout Pirayesh's career, and coached him to structure and submit this thesis under their guidance. As a result of their efforts and supported by the newly available long-term results, the place of Glyaderm® amongst other dermal regeneration matrices is firmly attested.

Aim and study set up.

The initial studies comprised of development of a dermal matrix from glycerol preserved allogeneic skin from conception to delineation of a prototype **(Chapter 2).**

The best prototype was named Glyaderm® (**Gly**cerolised **A**cellular **Derm**is) and compared with different dermal substitute matrices in a porcine wound model (**Chapter 3**) and as dermal scaffold for closure of abdominal wall defects in a rat model (**Chapter 4**).

Trial set-up and ethics approval for clinical trials necessitated us to investigate literature concerning skin replacement in burns (**Chapter 5**).

We performed a systematic review of scar assessment scales (**Chapter 6**) as well as a systematic review and critical appraisal of available scar assessment tools (**Chapter 7**) to update protocols for our clinical trials.

The first clinical publication of Glyaderm® showed favorable long-term results in 55 patients in a **two-stage procedure** (**Chapter 8).**

Collaboration with researchers from Nijmegen University resulted in visualization of newly synthetized collagen-elastin matrix *in vitro* and *in vivo* with Glyaderm® engraftment **(Chapter 9).**

A prospective, controlled, randomized, intra-individual, comparative, single -blinded study in a monocenter setting investigating the simultaneous application of Glyaderm® DRM + Split thickness skin graft vs autologous split thickness skin graft alone in full thickness skin defects and burns was performed with enrollment of 64 patients in a **one-stage procedure (Chapter 10)**.

We concluded our studies with an update on the evolution, scope, and future directives of Glyaderm® and its place amongst currently used DRM's (**Chapter 11**).

Outline

In order to improve wound healing of deep burns, dermal substitutes can be utilized in conjunction with expanded, thin autologous skin grafts. These dermal matrices can be sourced from either xenogeneic or human tissue, but antigenic structures such as cells and hairs must be removed to prevent adverse inflammatory responses upon implantation.

In this study, a cost-effective method for de-cellularizing human donor skin preserved in 85% glycerol using low concentrations of NaOH is described. Donor skin was incubated in NaOH for varying time periods of 2, 4, 6, or 8 weeks, and the resulting dermal matrix prototypes were analyzed using standard histology techniques. Functional tests were conducted in both rat subcutaneous implant and porcine transplantation models, where the prototypes were placed in full thickness excision wounds covered with autologous skin grafts **(Chapter 2).**

A porcine wound model was used subsequently to compare already known acellular dermal substitutes with our new prototype (Glyaderm) prepared from glycerol preserved human skin. All donor cells are removed by incubation in a solution of 0.06M NaOH. The dermal substitutes were applied to full thickness wounds and covered with an STSG. As a control, wounds were covered with only an STSG. The wound healing response was analysed for 8 weeks, macroscopically and on biopsies **(Chapter 3**).

To further evaluate the efficacy and biocompatibility of the "Glyaderm" dermal regenerative matrix (DRM), we conducted a subsequent study to assess its ability to provide coverage for abdominal wall defects. Abdominal wall repair can be performed using either synthetic or biological materials, with the latter often preferred due to their reduced risk of infections and fibrosis. In this study, we aimed to compare two acellular human dermis products using a rat model. One material was prepared using low concentrations of NaOH, while the other was the commercially available SureDerm®. Full thickness defects were created in the abdominal wall and repaired with the two materials. Rats were sacrificed at either 1- or 4-months post-operation, and the number of adhesions to the bowels were scored. Samples were collected for histological analysis and to measure the breaking strength of the repaired area. (**Chapter 4**).

We subsequently set out to review the literature on Skin Replacement in Burns. The aim of this study was to give an overview of which types of skin replacements have been developed and which problems still need to be faced. None of these

commercialized products can currently claim to be the optimal skin replacement, because clinical evidence is too scarce (several large multicenter trials are currently in process). The number of products becoming commercialized is nevertheless increasing steadily, which pleads for a certain overview, classification and clear comparison of the available products. **(Chapter 5).**

Due to the improvements in burn treatment as provided in highly specialized burn centers, more patients with deep and extended burn injuries do survive nowadays[16–18], resulting in a larger group of patients with more extensive scar formation[19]. Scar formation depends on several variables, including the wound treatment, the depth of the burn, the skin type and age of the patient, the healing process (inflammation, infection, etc.) but also on the application of preventive measures[20,21]. As a rule, wounds that are not healed within 2–3 weeks are considered most at risk for excessive scar formation[22].

Because of the relatively high prevalence of unfavorable scar formation after burns, most studies on scar assessment and scar treatment are focused on the burn scar[22–25]. Surgical and dermatologic scars will rarely result in extensive scar formation, and since the impact of scar complications strongly correlates with the dimension of the scar (e.g., pain, itching, and fragility), the impact of these types of scars is usually more limited, although also less well studied[26]. Therefore, burn scars are probably the scars with the highest impact on the quality of life[27–35]. Both physical and psychologic effects related to excessive scarring may hamper the quality of life, including the often lengthy, painful treatment, often resulting in still a suboptimal result[26,27,34,36–40]. Scars may cause pain, itching, and discomfort; and contractures may also constrict mobility. The integration of patients with hypertrophic scars in a society where well-being, individuality, and external appearance have become increasingly important might also be troublesome[27]. It has been demonstrated by many authors that burn scars, because of their clearly visible and stigmatizing appearance, may have a major psychologic impact, comparable to other chronic (skin) diseases[26,27,37,41–43]. A study of Balci et al. analyzed the quality of life in patients with hypertrophic scarring and keloids and found a similar impairment as in patients with psoriasis[37]. Brown et al. identified five main areas of impact in patients with excessive scarring resulting in coping behavior to hide or compensate the scars: the physical comfort and functioning, confidence in the nature and management of the condition, acceptability to self and others, social functioning, and emotional well-being[26]. They concluded that scarring has a major influence on a patient's psychologic morbidity and behavior and has important implications for clinical practice. Van Loey et al. described how scars may contribute to social anxiety and post-traumatic stress syndromes, since pressure garments or red and disfiguring scars can attract a lot of attention from other people, which may induce feelings of shame[27,44]. Several preventive measures and treatments have been proposed to decrease pathologic scar formation, and multiple invasive and noninvasive treatment modalities have been introduced[20,45–49]. Although scar assessment seems essential, this is still a neglected area, and there is still no consensus on the ideal method of scar evaluation, despite the many scales and tools that have been developed during the last

decades[49]. Adequate assessment of scars is, however, important in the clinical evaluation and follow-up, but it is also essential to compare different wound or scar treatment modalities[50–56]. Moreover, for medico-legal reasons, an objective scar evaluation can be required, e.g., for reimbursement of treatment and proof of disability.

Scar evaluation can be performed by rather simple, paper-and-pencil scar scales assessing several variables, usually by purely subjective word-descriptions (red, elevated, etc.), but also by using technically advanced and objective devices (scar tools) analyzing one or more variables in a more reproducible way (spectrometry, ultrasound etc.)[51,54,55,57–65]. The objective of this study was to provide an analysis and critical overview as to which scar scales have been developed to assess the physical aspect of burn scars, and what their role is in burn assessment **(Chapter 6**).

The paucity of literature on scar tools available for scar assessment brought us to investigate the available scar tools which can be used in burn scar assessment and research. **(Chapter 7**).

We extensively reported on the various cellular, acellular, temporary, and permanent skin replacements available for burns and full thickness defects in a previous publication[66]. Glycerol preserved acellular dermis (Glyaderm® - Euro Skin Bank, Beverwijk, The Netherlands) is the first non-profit dermal substitute derived from glycerol preserved, human allogeneic skin[66–68]. Glycerol preserved allogeneic skin (GPA) is routinely utilized as a temporary biologic dressing on partial thickness burns and as a means of wound bed preparation on excised burns. Allograft coverage prevents dehydration and infection of the wound and stimulates granulation formation to prepare the wound for closure with autologous skin[67,68]. Allografts contain donor cells, which are ultimately rejected and can therefore only be used as temporary wound coverage. Glyaderm®, which is decellularized by treatment with sodium hydroxide (NaOH), can be used to replace lost dermis in full thickness wounds serving as a dermal substitute. Glyaderm® consists of a collagen and elastin fiber network with native collagen and can ensure a bilayered skin restoration in combination with a thin autologous split skin graft. It is intended to be cost-effective and easy to use for widespread application in full thickness wounds such as full thickness burns. Glyaderm® is placed in a wound bed prepared with allografts, after which, a thin autologous split thickness skin graft (STSG) will close the wound following Glyaderm® ingrowth. Animal studies showed favourable results in terms of tissue integration and wound contraction and scar quality[68].

We first initiated a phase I pilot study to elucidate the most practical protocol for Glyaderm® application and to further investigate the scope of use of the dermal matrix in the clinical setting.

The second study was a phase III randomized, controlled, paired, intra-individual comparison of full thickness skin defects engrafted with Glyaderm® and STSG versus STSG alone in 55 patients with long term results **(Chapter 8).**

In tissue engineering and regenerative medicine, type I collagen is a critical biomaterial due to its significant role in the organization of tissues and organs and its involvement in organogenesis. Conversely, collagen gels are widely used in 3D

studies, especially in cancer research, to investigate cellular migrational behavior [70]. However, distinguishing between pre-existing collagen and newly synthesized collagen remains a significant challenge due to the highly conserved nature of collagens [71], which leads to cross-reactivity between different species. Current methods, including the use of antibodies, metabolic radiolabeling and mass spectrometry, are labour-intensive and do not provide topographical or organizational information about newly synthesized collagen fibers.

This study aimed to address this challenge by evaluating newly synthesized type I collagen using dermatan sulfate's intrinsic association with collagen fibrils. Proteoglycans decorin and biglycan, both collagen fibril-associated molecules that regulate collagen fibril diameter, contain dermatan sulfate, which remains associated with mature collagen fibrils [72,73]. The study utilized single chain variable fragment antibody GD3A127 to selectively detect dermatan sulfate combined with the absence of dermatan sulfate in experimentally or commercially produced biomaterials. The technique was tested using several collagenous biomaterials, including gels cultured with human fibroblasts with or without keratinocytes (denovoSkin and denovoDerm respectively)[74], experimental and commercially available scaffolds, and glycerol preserved acellular human dermis (Glyaderm®), both *in vivo* and *in vitro* **(Chapter 9).**

Although with the results of our previous studies it was concluded that Glyaderm® is a suitable replacement for the dermal layer in full thickness wounds, certain drawbacks limited a widespread application of this product. In the current era of universal budget restrictions, it is imperative to respect financial limitations when it comes to the implementation of new technologies. Burn care is already considered an expensive niche of our health care system. Costs are high because patients with burn injuries frequently require specialized treatment, prolonged hospitalization, intensive surgical and non-surgical treatment[75]. The initial surgical regimen for using Glyaderm® often consisted of three consecutive operations (allografts, Glyaderm® and STSG). This protocol increases the financial burden due to additional surgical procedures and the obligatory extended hospital stay of 3 weeks after implantation of the dermal substitute. Animal studies showed that simultaneous application of Glyaderm® and STSG was not feasible[76,77]. The dermal replacement should be able to supply nutrients to the STSG. This requires an adequate vascularization of Glyaderm® or the STSG will not survive. The research team attributed the impossibility of simultaneous application to the batch-to-batch inconsistencies and the proportions of Glyaderm®.

The Glyaderm® used in the previous studies proved to be too thick (thickness varying between 0.8mm to 1.3mm), obstructing rapid ingrowth of blood vessels, which is needed to vascularize the autograft. Glyaderm® with a more uniform and optimal thickness was needed. In the next phase selection by hand was performed, but as this was too labour intensive a laser tool had been developed. This purpose designed laser device can create Glyaderm® of a homogeneous thickness (0.30mm), resulting in a standardized Glyaderm® thus eliminating the batch-to-batch inconsistencies[78]. A pilot study with simultaneous application of Glyaderm® and STSG has already been

conducted. This pilot study has shown comparable results between the bilayered reconstruction and the STSG alone in terms of vascularization[79], graft take rate and wound healing time. Stronger evidence, based on a comparative intra-individual trial, of this simultaneous application was needed in order to prove its validity. Our research team has conducted a study to further investigating the simultaneous application of Glyaderm® and STSG in providing a bilayered skin reconstruction. The aim of this study is to gather the evidence proving that by reducing and standardizing thickness of Glyaderm® to 0.30mm, a simultaneous application of Glyaderm® and STSG is possible. Reducing the number of surgical interventions would not only make the application of Glyaderm® more cost-effective, but it would also decrease the morbidity since wound closure will be achieved one week earlier compared to the two-step procedure. We mentioned before that Glyaderm® is comparable to other currently available dermal equivalents, but with the advantage of being low-priced. Power calculation determined that 75 wound comparisons are to be included.

As hypothesis we state that treating deep burns and other full thickness skin defects with a simultaneous application of Glyaderm® + STSG will result in superior scar quality compared to the application of STSG alone.

This project started in October 2016. This study evaluates the results of the 80 wound comparisons of this study. This will include both short-term results (Glyaderm®/STSG take rate, microbial contamination, infection rate, length of hospitalization...) and long-term results gathered from follow-up (elasticity, erythema, water loss...). Patients are evaluated up to 12 months after complete wound closure. **(Chapter 10).**

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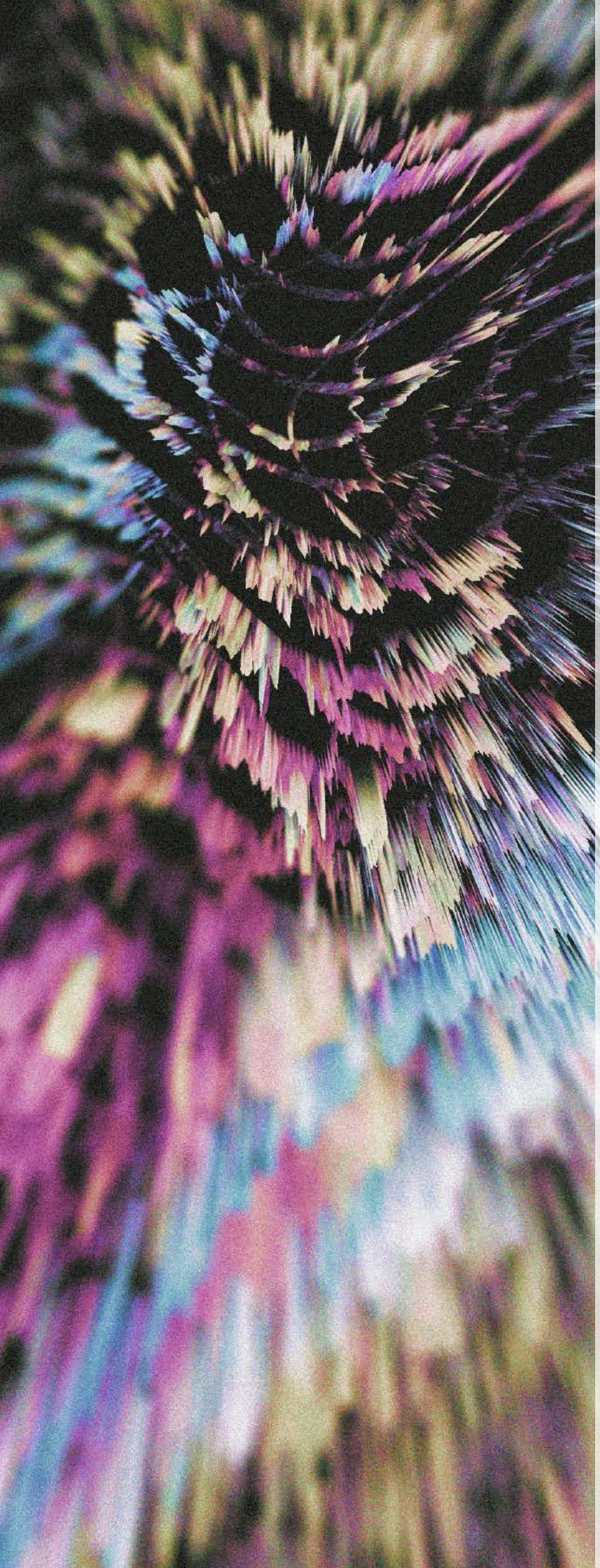

CHAPTER 2

DEVELOPMENT OF A DERMAL MATRIX FROM GLYCEROL PRESERVED ALLOGENEIC SKIN

CELL TISSUE BANKING (2008)

C. D. RICHTERS
A. PIRAYESH
H. HOEKSEMA
E. W. A. KAMPERDIJK
R. W. KREIS
R. P. DUTRIEUX
S. MONSTREY
M. J. HOEKSTRA

Abstract

Dermal substitutes can be used to improve the wound healing of deep bums when placed underneath expanded, thin autologous skin grafts. Such dermal matrix material can be derived from xenogeneic or human tissue. Antigenic structures, such as cells and hairs must be removed to avoid adverse inflammatory response after implantation. In this study, a cost-effective method using low concentrations of NaOH for the de-cellularization of human donor skin preserved in 85% glycerol is described. The donor skin was incubated into NaOH for different time periods; 2, 4, 6 or 8 weeks. These dermal matrix prototypes were analysed using standard histology techniques. Functional tests were performed in a rat subcutaneous implant model and in a porcine transplantation model; the prototypes were placed in full thickness exc1s1on wounds covered with autologous skin grafts.

An incubation period of 6 weeks was most optimal, longer periods caused damage to the collagen fibers. Elastin fibers were well preserved. All prototypes showed intact biocompatibility in the rat model by the presence of ingrowing blood vessels and fibroblasts at 4 weeks after implantation. An inflammatory response was observed in the proto- types that were treated for only 2 or 4 weeks with NaOH. The prototypes treated with 6 or 8 weeks NaOH were capable to reduce wound contraction in the porcine model. In neo-dermis of these wounds, elastin fibers derived from the prototype could be observed at 8 weeks after operation, surrounded by more random orientated collagen fibers. Thus, using this effective low-cost method, a dermal matrix can be obtained from human donor skin. Further clinical studies will be performed to test this material for dermal substitution in deep (burn) wounds.

Introduction

Advances in intensive care have resulted in decreased mortality and morbidity, especially with major bums. The current focus in bum care has shifted towards improving the long-term function and appearance of the healed skin in conjunction with quality of life.

This focus on quality has generated a significant amount of research into the use of skin substitutes to control pain, to enhance wound closure and for a better functional and cosmetic outcome. The bilayer concept of wound coverage in which both epidermal and dermal analogs are used is gaining widely accepted[1] . The outer layer of such substitutes must have a barrier function to protect the wound not only from bacterial contamination, fluid loss, but also, overheating and accumulation of tissue fluids. Dermal elements are important for cell guidance during granulation tissue formation, re-modelling and re-epithelialization. The dermis is essential for restoring normal tissue architecture, diminution of wound contraction and for the prevention of scars. Wound healing outcome of thin split skin grafted deep burn wounds is often poor, with respect to elasticity and cosmetic appearance due to the lack of a significant dermal component. Paucity of available autograft in major burns

necessitates wide mesh grafting which can result in excessive granulation within the interstices and delayed wound healing leading to increased scar formation. Application of a dermal substitute underneath the autologous skin is a possibility to improve the wound healing process[2,3]. Dermal substitutes can be derived from xenogenic tissues (mainly pig or bovine) or human skin. In both cases, all cells and hairs must be removed in such way that the structure of the collagen and elastin fibers is preserved. De-cellularized human donor skin should ideally provide a structurally intact natural three-dimensional collagen and elastin matrix. Human skin can be procured from donors after medical screening and serology tests. Donor skin is available from skin banks. The Euro Skin Bank in Beverwijk, the Netherlands, preserves donor skin in 85% glycerol. This is a simple, cost-effective method resulting in non-viable but intact skin that can be used as biological dressing on scalds, temporary coverage on excised burns and as a means of wound bed preparation[4]. All cells are nonviable, but the collagen and elastin fibers are well preserved using this method[5]. In addition, glycerol 85% is a slow but effective in-activator of microorganisms[6–8].

The method to remove antigenic structures, such as cells from the tissue should be effective without interference with the biocompatibility. Blood vessels and fibroblasts must be able to migrate into the processed tissue after implantation. Ideally, these cells attach to the matrix molecules and will integrate the dermal substitute with the autologous skin placed on it, in this way reducing the contraction of the wound during healing. Within time, the donor collagen will be removed by the cells of the recipient and replaced by new collagen. We hypothesize that the elastin fibers present in donor skin will not be replaced but will serve as “guidance” for the ingrowing blood vessels and fibroblasts. This will result in a more randomly organized new collagen with higher pliability. NaOH has been used in the processing of bovine and porcine tissue by Bioplex B.V. (Datascope) to obtain membranes suitable to serve as a template for the formation of neodermis[9,10]. This is an effective and low-cost method to remove donor cells and hairs. In the present study, we have used a slightly modified method with lower concentrations of NaOH. Human skin was processed for 2, 4, 6 or 8 weeks in the NaOH solution. The obtained prototypes were analysed using histology and were tested in two different animal models to evaluate their biocompatibility and function as dermal matrix.

Methods

Preparation of the prototype samples from human skin

Donor skin preserved in 85% glycerol was obtained from the Euro Skin Bank, Beverwijk, The Netherlands.

After removal of the glycerol by repeated washings in 0.9% NaCl, the skin was incubated in 0.06 N NaOH. Incubation was performed for 2, 4, 6, 8 weeks with agitation, every week the NaOH solution was replaced. After the treatment with NaOH, neutralization was done using 0.1 N HCI for 10 min, followed by washing in

0.9% NaCl. The obtained materials were preserved into 85% glycerol until use in the experiments. Before use, the glycerol was removed by incubation in 0.9% NaCl. NaOH treatment) were taken for histological examination and were tested in two animal models. Subcutaneous implantation to evaluate biocompatibility was performed in a rat model. The different prototypes were also used as a dermal matrix underneath a meshed split skin graft in a porcine full thickness wound model, to test the effects on the outcome of the wound healing process.

Animal models

Rat implantation model

Animals

Rats were obtained from Harlan CPB (Zeist, The Netherlands). A total of 16 male WAGRy rats (8-10 weeks) were used for the experiments, their weight was 200-220 g at the time of operation. The Animal Welfare Committee of the VU Medical Centre has approved the research protocol. The rats were kept under routine laboratory conditions with free access to water and food. After operation, rats were kept in separate cages.

Experimental procedure

Rats were anaesthetized using a mixture of Hypnorm® (fentanyl, Janssen, Berchem, Belgium) and Dormicum® (Midazolam, Roche, Mijdrecht, The Netherlands), injected subcutaneously. After shaving, an incision of 1.5 cm was made up to the subcutaneous fat layer and a pocket of one by 1 cm was created using scissors. A sample of the prototype of one by | cm was placed in this pocket. The incision was sutured with 4.0 Vicryl.

Four groups of rats (four animals per group) were implanted with the 2, 4, 6 or 8 weeks in NaOH processed prototype. The rats were sacrificed at 4 weeks after operation by O_2/CO, exposure and after macroscopic inspection; the implants with surrounding tissue were excised and placed in Kryofix solution (ethanol/PEG 400; E. Merck, Darmstadt, Germany). Samples were further processed for histology.

Porcine full thickness wound model

Animals

Four female Yorkshire pigs (weight 30- 35 kg) were used for the experiments. The Local Animal Welfare committee of the VU Medical Center approved the treatment protocol according to the Dutch law on animal experiments.

Experimental procedure

On week before operation, a grid was tattooed by cutting the skin with a scalpel till sub-epidermal depth and applying tattoo ink. Four full thickness excision wounds of

4 x 4 cm on each flank were prepared under anaesthesia. The prototypes (2, 4, 6 or 8 weeks treated with NaOH) were placed into the different wounds, thus on each pig each prototype was tested 2x. Dermal matrix materials were meshed 1:1.5 for wound drainage only and sutured with a closed mesh pattern into the wound bed. Autologous split skin was recovered from the back of the animal using a dermatome (Aesculap, Germany). The autologous split skin was meshed 1:3 and was sutured with an open mesh pattern on top of the dermal matrix with SurfaSoft (Derma-prof, Moerkapelle, The Netherlands). Wounds were dressed with 0.9% NaCl soaked cotton gauzes that were fixed using adhesive bandages (Curafix, Lohmann & Rausher, Almere, The Netherlands). The dressings were protected from mechanical disturbance using an elastic bandage (Tubigrip, Medeco, Oud-Beijerland, The Netherlands).

The wound dressings were changed on day 4 and 7 after operation, thereafter weekly until all wounds were closed. Surfasoft was removed at day 7 and the take of the autologous skin was scored. Biopsies (4mm) were taken at day 7, 14, and 21 after wounding. Wound contraction was measured by tracing the edges of the wound and the tattoo grid on transparent film. Contraction was measured using planimetry and expressed as a percentage of the original wound area, corrected for the growth of the animals. Digital photographs were taken for macroscopic wound healing evaluation. Animals were sacrificed at 8 weeks after operation. After macroscopically inspection and planimetry, biopsies covering the full width of the wound were excised, placed in Kryofix and further processed for histology.

Immuno-histochemistry

Sections of 5 um thickness were cut and stained with the following methods:

- Haematoxilin-Eosin (Gurr, BDH Ltd, Poole, UK), for standard histology.
- Elastica von Giesson (Merck, Darmstadt, Germany), to stain collagen and elastin.
- α-smooth muscle actin (αSMA) antibody (Sigma) staining. Pericytes, which are present in blood vessels, and myofibroblasts are recognized by this antibody.

After fixation in acetone, slides were incubated with the αSMA antibody for 45 min at room temperature. Thereafter the slides were washed three times with PBS followed by incubation with a secondary antibody conjugated with horseradishperoxidase (rabbit anti mouse, Dako, Glostrub, Denmark) for | h at room temperature. After washing with PBS, slides were incubated with diaminobenzidine (Dako) to visualize the positive cells (dark brown colour). Two independent observers analysed the sections. The outgrowth of the epithelial cells of the autologous split skin graft was scored on the haematoxylin-eosin (HE) stained sections using a grid with a fixed area in the ocular of the microscope. The inflammatory response was scored on the HE sections using a grid within the ocular of the microscope measuring 1 mm^2 (10 by 10 fields). The areas covered with clusters of inflammatory cells (i.e., neutrophilic granulocytes, monocytes and

lymphocytes) were counted. In addition, vascular ingrowth was assessed on the sections stained with the αSMA antibody. The presence of elastin fibers and the orientation of the newly formed collagen fibers were studied on the sections stained with Elastica von Giesson.

Statistical analysis

The results (outgrowth of epithelial cells and inflammatory response) were analysed for significance using the non-parametric Kruskal-Wallis test. A P-value below 0.05 was considered to be statistically significant.

Results

Histological examination of the samples

Figure 1 shows the pattern of an intact collagen elastin matrix in a prototype treated for 6 weeks with NaOH. The intact elastin network contains structurally important microfibrils. We observed slight damage to the collagen in the prototypes that were treated for 8 weeks with NaOH.

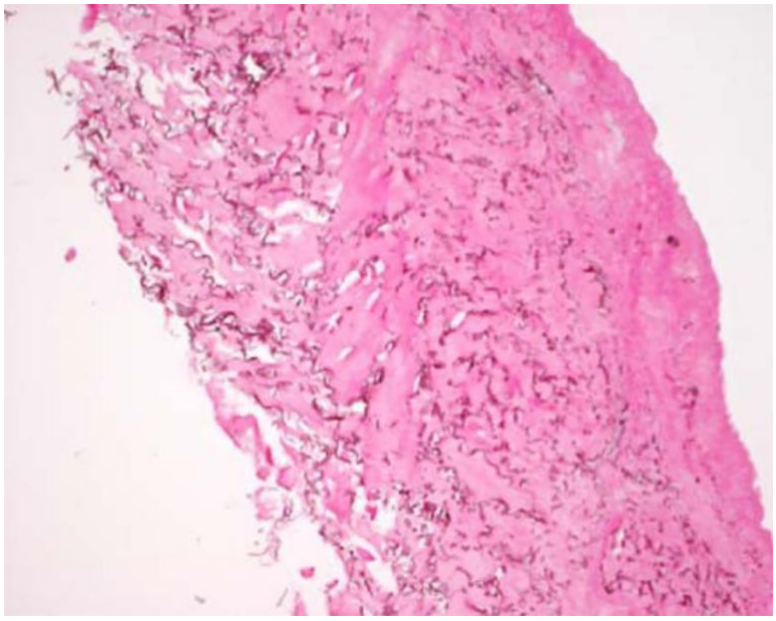

Figure 1. Section of a prototype dermal substitute treated for 6 weeks with NaOH, stained with Elastica von Giesson staining. Dark fibers are elastin fibers.

Rat implantation model

None of the rats had complications, such as wound infection to the implantation site. The implanted tissues were structurally intact and integrated with the surrounding tissue at 4 weeks after operation.

On sections of the implants, inflammatory cells were observed in the prototypes that were treated with NaOH for 2 and 4 weeks. This was less pronounced in the

prototypes treated for 6 or 8 weeks with NaOH. Fibrovascular ingrowth was present in all implants, but more vessels were present in the implants with inflammatory cells (**Figure 2**). The implants that were treated with NaOH for 8 weeks showed more amorphous collagen structure and some damage to the fibers could be observed.

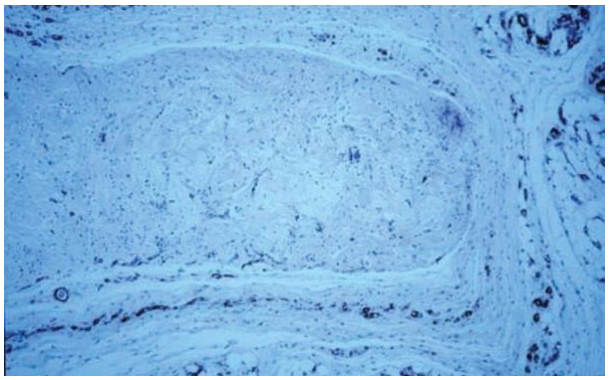

Figure 2. Representative picture of rat tissue showing an implant, treated 6 weeks with NaOH, 4 weeks after operation. Ingrowth of blood vessels (dark brown stain) and fibroblasts (blue cells) is present.

Porcine full thickness wound model

The take of the meshed autologous split skin grafts placed on the different prototypes did not show much difference when scored at day 7 after operation. Between 65 and 80% (average 75%) of the autologous skin had survived the first days after operation when granulation tissue and new vessels have to grow from the wound bed into the graft.

At day 14 after operation however, clear differences were present between the prototypes. The autologous skin grafts placed on the 6 or 8 weeks NaOH treated prototype has reached 100% wound closure, whereas on the 2 and 4 weeks NaOH wounds still showed lesions (**Figure 3**). Outgrowth of the epidermis is significant delayed in these latter wounds ($P < 0.05$, Kruskal-Wallis test). All wounds were closed at day 21.

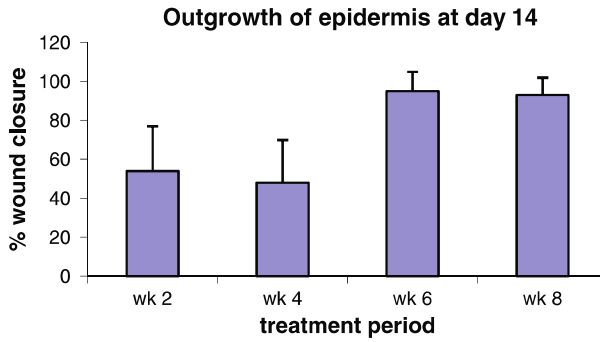

Figure 3. Porcine excision wounds transplanted with the different prototypes and covered with autologous skin (meshed 1:3). The percentage of the wound bed covered with epithelial cells was counted on biopsies of the wounds at day 7, 14, and 21. At day 14, outgrowth of the autologous skin is faster on prototypes treated for 6 or 8 weeks with NaOH, resulting in earlier closure of the wound (significant difference in percentage wound closure between wounds treated with the 2 or 4 weeks processed prototype and wounds treated with the 6 or 8 weeks prototype, $P < 0.05$, Kruskal-Wallis). Shown are the means (-+S.D.) of eight wounds per treatment.

On the sections of biopsies taken at day 14, it appeared that the number of inflammatory cells was significant higher in the prototypes treated for 2 or 4 weeks with NaOH (**Figure 4**, $P < 0,05$, Kruskal-Wallis test). This delay in outgrowth of the autologous skin may be caused by this inflammatory response.

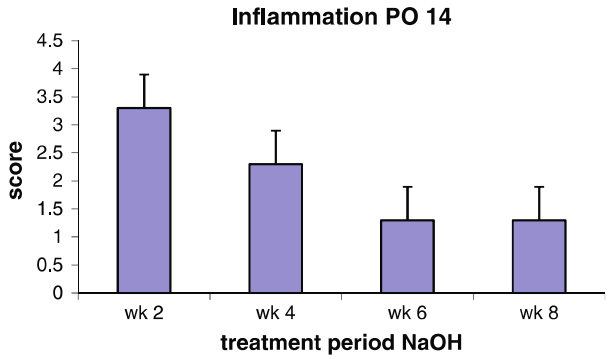

Figure 4. Longer treatment periods with NaOH reduced the amount of inflammation in the prototype after implantation under autologous skin grafts in excision wound (porcine model). At day 14 after operation, the number of inflammatory cells was significantly higher in the prototype processed for 2 or 4 weeks compared to the 6 or 8 weeks processed prototypes. Data are expressed as the mean (+S.D.) of eight wounds for each treatment.

At 8 weeks after operation, wounds transplanted with the 6 or 8 weeks NaOH treated prototype showed the best results with respect to scar formation and wound contraction. The human derived collagen of the implants could be easily distinguished from newly produced porcine collagen; the implant fibers are thicker and larger.

Most of the collagen of the prototypes was replaced by newly produced collagen and donor derived elastin fibers were still present, especially in the wound treated with the 6 or 8 weeks NaOH processed prototype. The new collagen fibers showed a more parallel orientation in wounds treated with the 2 or 4 weeks NaOH processed prototype, whereas in the wounds treated for 6 and 8 weeks randomly organized fibers around the elastic fibers were present (**Figure 5**).

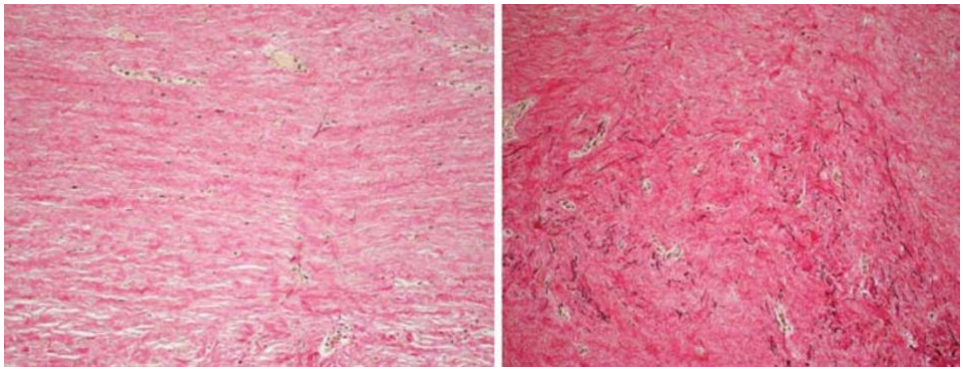

Figure 5. Sections of the scar tissue, stained with Elastica von Giesson. The sections were prepared from biopsies taken at 8 weeks after operation. The prototypes week 6 (right picture) and week 8 resulted in a scar with more randomly organized collagen fibers. Elastin fibers are black, most probably originating from the prototypes, surrounded by new porcine collagen. Left picture: biopsy of a wound treated with a prototype that was treated for 2 weeks with NaOH.

Discussion

The NaOH de-cellularization method can be an effective method to remove donor cells and hairs from human donor skin. The optimal incubation time with a low concentration of NaOH is 6 weeks. The prototype treated for 2 or 4 weeks induced an inflammatory infiltrate after implantation and longer periods (>8 weeks) caused clearly visible damage to the extra cellular matrix. The obtained materials still show biocompatibility as demonstrated in the rat model, by the ingrowth of fibroblasts and blood vessels. The number of vessels was the lowest in the 8 weeks NaOH prototype, at 4 weeks after implantation. In general, we observed more blood vessel ingrowth in prototypes with higher numbers of inflammatory cells. Experiments using rabbits with Alloderm®[11], an acellular human dermis product of Life Cell Corporation (Branchburg, NJ, USA) placed in subcutaneous pockets showed that within 2 weeks the implant was completely penetrated with blood vessels. Our NaOH method to obtain acellular human dermis is a low-cost method and possibly more stringent compared to Alloderm®, removing more antigenic elements. After processing in NaOH, the dermal matrix tissue can be preserved in 85% glycerol again until use which makes it easy to store without further damage to the collagen and elastin fibers that might be caused using cryopreservation or freeze-drying techniques.

The results from the porcine excision wound model indicate the 6 or 8 weeks NaOH treated prototype can serve as a functional dermal substitute; wound contraction was

reduced, and the newly synthesized collagen bundles showed a more favourable orientation. We observed clear differences between the four prototypes. Treatment with NaOH for 2 or 4 weeks was apparently too short to remove all antigenic components; an inflammatory response was induced that most probably interfered with the outgrowth of the epidermis from the autologous skin graft. We have observed earlier in other experiments that inflammatory cells can slow down keratinocyte outgrowth; in a rat partial excision wound model[12] but also in full thickness wound in a porcine model[13]. These wounds were grafted with the sandwich technique; meshed autologous skin covered with fresh allogeneic skin or glycerol preserved allogeneic skin. The fresh, untreated allogeneic skin induced higher numbers of macrophages and T cells to the wound bed and the outgrowth of the autologous skin was completed 3 days later compared to wound covered with glycerol preserved allogeneic. The influx of inflammatory cells may have resulted in earlier breakdown of the collagen and elastin in the 2 or 4 weeks NaOH treated prototypes. Macrophages and neutrophils play an important role in the removal of bacteria and debris in the wound bed. However, their abundant presence in the wound bed may result in damage to the dermal substitute or migrating epithelial cells due to their proteolytic enzymes[14,15]. We observed in the sections of the biopsies new collagen produced in an orientation parallel to the epidermis and wound bed suggesting that infiltrating host fibroblasts were not able to use the prototype as scaffold. In the wounds treated with the 6 or 8 weeks NaOH prototype however, we observed in the sections the fibroblasts could attach to the human tissue derived elastin fibers and synthesize the new fibers around these fibers.

It must be taken into account however that the human derived prototypes are tested in xenogeneic animal models. Walter et al. have reported two other methods to obtain a-cellular dermal matrix from human or porcine skin using NaCl-SDS or dispase-Triton X-100[16]. When tested in a rat wound model, the porcine a-cellular dermal tissue showed poor results with respect to graft takes and wound contraction, whereas rat a-cellular dermal tissue was functional[17,18]. Most likely, there are more differences between rat and porcine tissue compared to human and porcine tissue, but we observed also reduced survival of the autologous skin graft on the prototypes that were treated for 2 or 4 weeks with NaOH. In contrast, we show improved results with respect to outgrowth of the epidermis when the prototypes were treated for a longer period (6-8 weeks) with NaOH. Thus, it is possible to evaluate how the method of processing influences the host response after implantation in this model.

Elastin is an extracellular matrix protein that provides elasticity to tissues and organs. It is abundant in organs where elasticity is of major importance, such as blood vessels and skin. The highly specialized elastin matrix in which elastin microfibrils are incorporated and assembled is a key component for skin elasticity; we show that this matrix structure is well preserved using the de-cellularization method with NaOH on glycerol preserved donor skin. In addition, our results indicate that these donor-derived elastin fibers can be important for the ingrowth of host blood vessels and fibroblasts and in this way will “guide” the fibroblasts in the turnover of donor collagen into host collagen leading to a more natural newly formed dermis.

Thus, in conclusion, using donor skin, a functional dermal substitute based on the natural structure of human skin can be prepared using a cost-effective NaOH de-cellularization method. We advocate further clinical assessment of this dermal substitute (stored in glycerol, Glyaderm) in combination with autologous skin for effective bilayered skin restoration.

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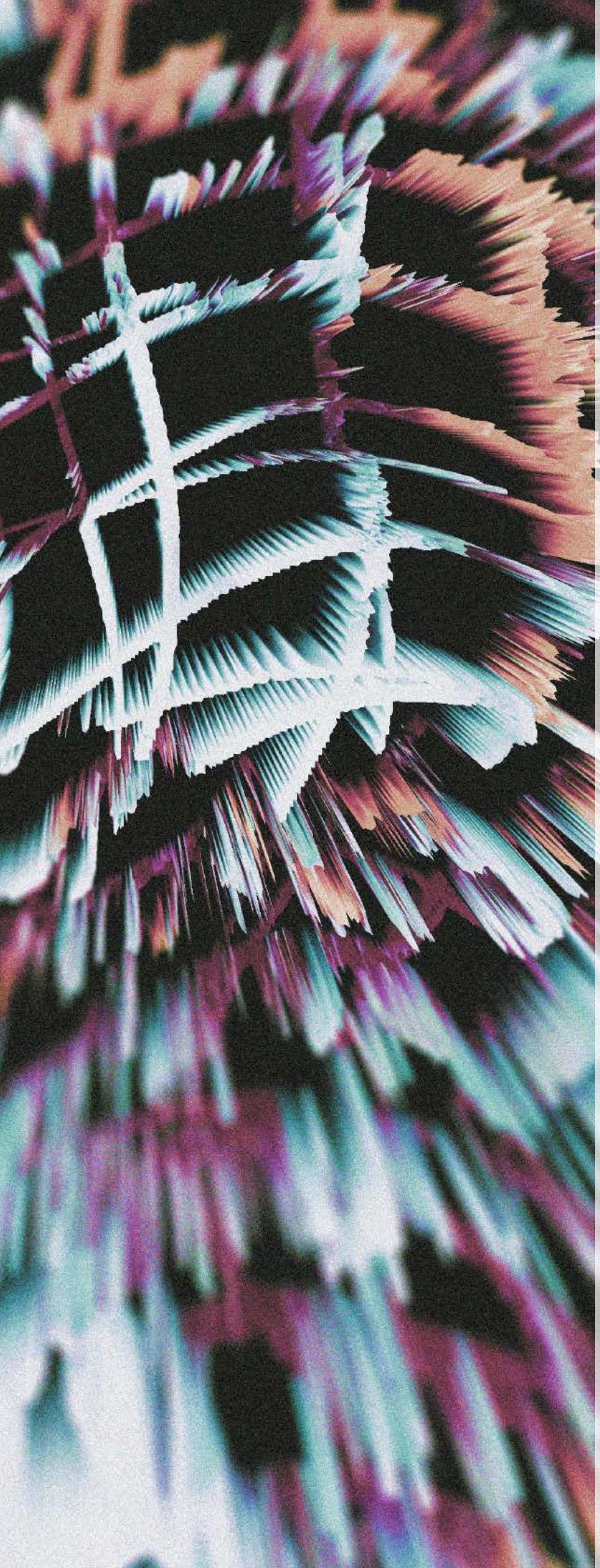

CHAPTER 3

COMPARISON OF GLYADERM WITH DIFFERENT DERMAL SUBSTITUTE MATRICES IN A PORCINE WOUND MODEL

JPRAS OPEN (2022)

A. PIRAYESH
I. DE DECKER
C.D. RICHTERS
N.J. PAAUW
H. HOEKSEMA
M.J. HOEKSTRA
K.E.Y. CLAES
B. VAN DER LEI
S. MONSTREY

Abstract

Background

Closure of extensive burn wounds with widely expanded autologous split-thickness skin grafts (STSG) is associated with undesirable scar formation and contraction, due to the lack of dermis. Various materials for dermal replacement have been developed, either of xenogeneic, allogeneic or synthetic origin and are placed in the wound underneath a thin STSG in order to improve scar quality. In this study a porcine wound model was used to compare several commercially available acellular dermal substitutes with a acellular dermal substitute prepared from glycerol preserved human skin: Glyaderm®.

Methods

Antigenic components of the allografts were removed by incubation in 0.06M NaOH solution. In the first experiments, the dermal substitutes were applied to full thickness wounds and covered simultaneously with STSG. Controls were covered with STSG only. The wound healing response was analysed for 8 weeks, both macroscopically and histologically. The Mann-Whitney U test was used for statistical analysis. In the second series of experiments Glyaderm® was applied in a two-stage procedure in comparison to Integra. The STSG was placed on the dermal substitutes one week later.

Results

In the first series, the inflammatory response and myofibroblast influx in Glyaderm® was limited, indicating possible beneficial outcomes on final wound healing results. Survival of the STSG on the acellular dermis was lower compared to the control wounds. Second series: the take of the STSG was the same as in the controls, but additionally wound contraction was reduced. Application of Glyaderm® was non-inferior to Integra.

Conclusion

Glyaderm® can be successfully used for the reduction of wound contraction when applied in a two-stage procedure.

Background

Extensive full thickness burn wounds can be closed with widely expanded autologous split-thickness skin grafts (STSG) after debridement. The results obtained with this standard surgical technique are less favourable in terms of contraction and scar quality, mainly due to the lack of dermis. Several materials for dermal replacement have been developed. These substitutes can be placed underneath the STSG and serve as a scaffold into which cells can infiltrate and repair the wound, ultimately resulting in less scar tissue formation and contractures.

Nowadays, different dermal substitutes such as Alloderm®, Matriderm® and Integra are available on the market, but the benefit and cost-effectiveness of these materials is still under discussion[1-4]. Alloderm® (Lifecell Corp., Branchburg, NJ) is an acellular dermal substitute processed from cryopreserved human cadaver skin. When combined with a very thin STSG, the take rate of Alloderm® was improved and in the long term less scarring and contractures were reported[5]. Integra (Lifesciences Corp, Inc, Plainsboro, NJ).) consists of cross-linked bovine collagen and chondroitin-6-sulfate covered with a silicone layer to temporarily provide wound coverage. Integra is applied during the first operation after debridement and preparation of the wound bed. After a period of 2 to 4 weeks, the silicone layer is removed during a second operation and autografting is performed. The silicone layer serves as a barrier against bacteria, it controls water evaporation and provides mechanical support. Several studies using Integra have reported less hypertrophic scar formation, but also increased risk of infection[6]. Matriderm, another commercially available dermal substitute, consists of a lattice of bovine collagen coated with elastin hydrolysate. Promising results were obtained, but in the long term no significant difference was observed when compared to wounds treated with STSG only except for a less visible meshed scar pattern[7]. The possibility of Matriderm®to be used in a single-step procedure is a practical advantage[8] but comparative clinical data are limited. In a small clinical trial[9] improved scar elasticity was observed in the Matriderm-group combined with sheet autografts compared to wounds treated with sheet autografts only.

To achieve optimal results, a substitute requires low antigenicity, stability as a dermal template and the capacity for rapid vascularization to ensure survival of the overlying STSG. Dermal substitutes can be derived from xenogeneic tissue, allogeneic tissue from human skin or synthesized with acellular materials from synthetic sources. Decellularized human donor skin ideally provides the natural three-dimensional collagen and elastin structure. All cells and appendages have to be removed in such way that the structure of the collagen and elastin fibers is preserved. Several methods to remove antigenic structures have been described, using sodium dodecyl sulphate (SDS) and freeze drying techniques (Alloderm®, Lifecell [5,10]) or Triton X-100 combined with Dispase[11]. In the present study, we evaluated the use of a human dermal matrix prepared from glycerol preserved allograft skin (Glyaderm[12]) using low concentrations of NaOH in a porcine wound model. This NaOH treated dermal

substitute was compared to Integra, Alloderm® and de-epidermized acellular dermis (DED)[13-15].

Methodology

Substitute materials and animals

Ethical clearance

Human participants were not included. The experiments with animals were approved by the animal welfare committee of the Vrije Universiteit Medical Centre, Amsterdam. Human skin was obtained from donors with consent according to the Dutch Law on Organ donation.

Dermal substitutes

Glyaderm® was prepared from donated human skin by the Euro Skin Bank as described earlier[12] using low concentrations of NaOH (0,06 M). DED was prepared according to the method in literature[12-14] using incubation in phosphate buffered saline (PBS). Acellular dermal tissue was prepared by repeated washing of glycerol preserved donor skin (Euro Skin Bank, Beverwijk, the Netherlands) in sterile PBS supplemented with 50 IU/ml-1 penicillin G; 50 µg/ml-1 streptomycin (Gibco, Paisley, U.K.), for 3 weeks at 37°C and further stored at 4°C (no longer than 6 months). Alloderm® was ordered from Lifecell (Lifecell Corp., Branchburg, NJ) and Integra from Lifesciences (Lifesciences Corp., Inc, Plainsboro, NJ).

Animals

Twelve female Yorkshire pigs (weight 30-35 kg) were used. The same animal model as described earlier[12]. A grid was tattooed one week prior to the first operation, by cutting the skin with a scalpel till sub-epidermal depth and applying tattoo ink, allowing measurement of wound contraction and applying a natural growth correction.

Experimental procedures

This study was conducted in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

One stage procedure

Four full thickness excision wounds of 4x4 cm were inflicted on each flank of the animals under general anaesthesia. The dermal matrices that were tested (Glyaderm®, Alloderm® and DED) were meshed 1:1,5 and sutured into the wounds. An Aesculap dermatome (B. Braun) was used to harvest autologous split skin (0.2-0.3 mm

thickness) from the animal's dorsum. The autologous skin was meshed 1:3 and sutured on top of the dermal matrix. Finally, skin grafts were covered with SurfaSoft (Haromed, Ghent, Belgium). Further wound dressing was performed as described earlier[12].

Two-stage procedure

The animal model was similar to 2.2.1, again 4 full thickness excision wounds of 4x4 cm were prepared under anaesthesia on each side of the animal, but then the control wounds were directly covered with STSG meshed 1:3. Glyaderm® and Integra were meshed 1:1,5 and sutured into the wounds with Surfasoft on top of the Glyaderm®. Further wound dressing was performed as described earlier[12]. Seven days later, the second operation was performed. STSG's were harvested from the dorsum, meshed 1:3 and sutured on top of the dermal substitutes.

Evaluation of wound healing

Dressings were changed on day 4 and 7 post-surgery and assessed for any signs of infection. Thereafter, wound dressings were weekly changed until the wounds were completely closed. We removed Surfasoft at day 7 and the take rate was assessed. Biopsies (4mm) were taken at day 7 and 14. Wounds colonized with bacteria were excluded from analysis.

The pigs were sacrificed at 8 weeks after surgery. After macroscopic inspection of the scars and measurement of wound contraction using planimetry, we excised large biopsies covering the full wound. Digital photographs were taken to evaluate wound healing evaluation at day 7, 14 and day 56.

Planimetry

The planimetry was performed as described in earlier studies[12]. Briefly, wound contraction was measured by tracing the edges of the wound and the tattoo grid on transparent film. Visitrak was used to measure contraction.

(Immuno)-histochemistry

Sections were prepared of 5 μm thickness and stained using the following methods:

1. Haematoxilin-Eosin (Gurr, BDH Ltd, Poole, UK), for standard morphology of the wounds and the cells present.
2. Elastica von Giesson (Merck, Darmstadt, Germany), to stain collagen and elastin.
3. 3. α-smooth muscle actin (α-SMA) antibody (Sigma) to stain pericytes and myofibroblasts, which are present in blood vessels and scars respectively.

After 10 min fixation in acetone, slides were incubated with the sections with the α-SMA antibody diluted in PBS for 45 min at room temperature. Thereafter the slides were washed thrice with PBS followed by incubation with a secondary antibody

conjugated with horseradish-peroxidase (rabbit anti mouse, Dako, Glostrub, Denmark) for 1 hour at room temperature. After a washing step using PBS, the slides were incubated with diaminobenzidine (Dako) to stain the positive cells.

Two independent observers analysed the stained sections and scored the influx of cells. The haematoxylin sections were used to analyse the influx of inflammatory cells. In the biopsies taken at the end of the experiment, an ocular grid was used in the microscope to quantify the areas in the dermal matrices with inflammatory cells. Myofibroblasts were quantified on digital images of the stained sections and analysed using Lucia G software.

Statistical analysis

Statistical analysis was performed using Graphpad Prism version 9.0.2 (San Diego, CA, USA). Normality of data was assessed using the Shapiro-Wilk test. Significant differences between treatments were assessed with the Mann-Whitney test. A priori, values of $p < 0.05$ were considered to be statistically significant.

Results

One stage procedure

Survival of the autologous split thickness skin graft

The Surfasoft was removed at day 7 after operation from the wounds and the take rate was scored as the percentage that was still viable. **Table 1** shows that the take rate on a dermal substitute in a one-stage procedure is lower compared to the control wounds. AlloDerm® showed the best result, followed by Glyaderm® and DED.

	Number of wounds	Mean take rate*	Range take rate
Control	12	92	75-100
Glyaderm®	12	65	25-100
Alloderm®	6	75	35-100
DED†	8	50	20-100

Table 1. Take rate of autologous split skin in the one stage procedure. *The percentage of the autologous skin that was still viable (take rate) at day 7 after operation is higher in control wounds with no acellular dermis. †De-epidermized acellular dermis.

Inflammatory response in the dermal matrices

In the sections of the biopsies from day 7 and 14, numerous immune cells were seen in the dermal matrices compared to the control. The highest numbers seemed to be present in the DED, followed by Alloderm®. The number of cells that could be observed in the Glyaderm® matrix was relatively low but higher in comparison with the control wounds. In the sections from the biopsies taken 8 weeks after operation,

the matrices could be observed in the formed scar tissue. Elastin fibers surrounded with large collagen fibers were present that could be easily distinguished from the new thin fibers produced by fibroblasts migrated into the matrices. Macrophages and lymphocytes were also present in the dermal matrices, sometimes large accumulations were observed, especially in the DED matrix. Since the week 8 sections were from biopsies covering the whole wound area, it was possible to quantify the areas within the dermal matrices covered with inflammatory cells. As can be seen in **Figure 1**, the inflammatory response was significantly higher in DED and Alloderm® compared to Glyaderm®.

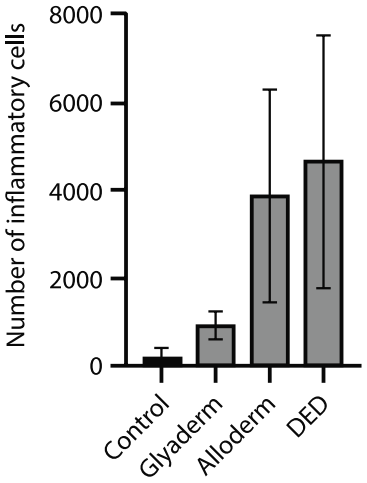

Figure 1. The influx of inflammatory cells in the scar at day 56 is higher when acellular dermis is applied to the wound in comparison to controls. In addition, the type of acellular dermis has a clear effect on the inflammatory response.

Eight weeks after surgery, the area with inflammatory cells in Glyaderm-treated scars was significantly lower compared to wounds treated with Alloderm® or DED ($p < 0.05$).

Effect of the matrices on scar formation and wound contraction

In case the take of the autologous skin on the Glyaderm® was > 70%, the quality of the scar seemed better (**Figure 2**) but there were no significant differences in wound contraction between controls and wounds treated with a dermal substitute in the one-stage procedure.

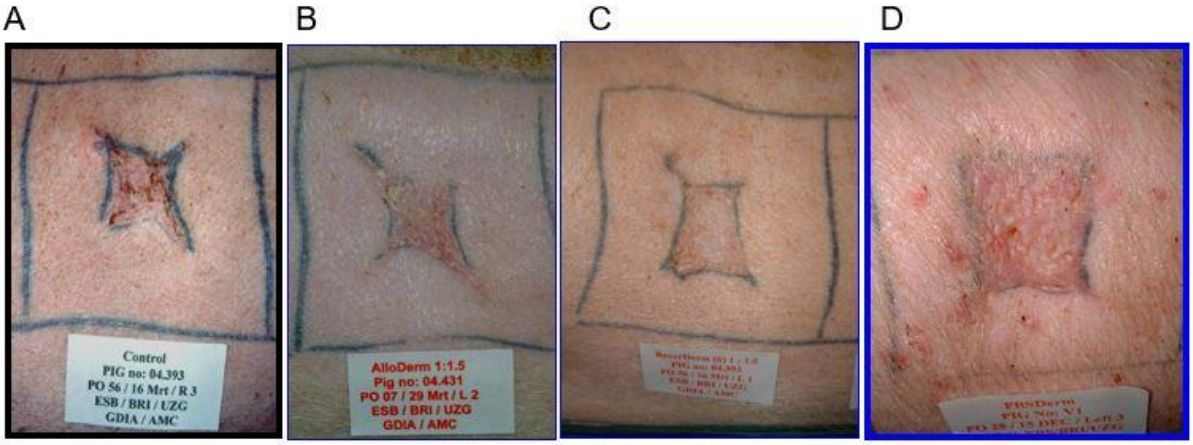

Figure 2. Macroscopic aspects of control wound (A) and wounds treated with Alloderm® (B), Glyaderm® (C) or DED (D). Pictures of representative scars at 56 days after injury. Wounds treated with Glyaderm® scored higher in terms of absence of erythematous appearance. Wounds with a take > 75% treated with Glyaderm® had the best score with respect to colour and smoothness. No significant differences in wound contraction were observed between groups at 8 weeks after operation.

Effect of the matrices on the numbers of myofibroblasts

Myofibroblasts are associated with wound contraction and hypertrophic scarring[16-17]. Although the sections from biopsies of the control wounds showed high numbers of strongly positive myofibroblasts in the new scar tissue, this was not significantly different compared to wounds treated with a dermal substitute. There were also no significant differences in myofibroblast numbers between wounds treated with Glyaderm®, Alloderm® or DED (**Figure 3**).

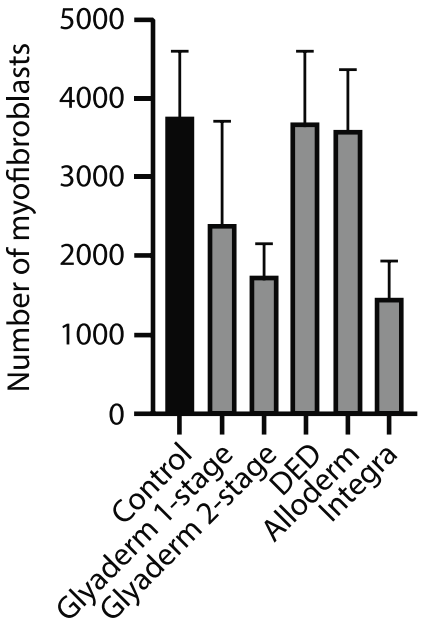

Figure 3. Application of Glyaderm® or Integra in a two-stage procedure resulted in lower numbers of myofibroblasts in the scar (week 8).

Two stage procedure

Effect of a two-stage procedure on graft survival

Glyaderm® was tested in a two-stage procedure to optimize graft take. Autografting took place 1 week post-implantation. After dressing removal, the Glyaderm® had a slightly red appearance if no infection or dehydration had taken place. The thickness of Glyaderm® had to be < 0.6 mm to enable blood vessel ingrowth within one week.

The take rate of the autologous skin on the dermal substitute is much higher compared to the one stage procedure (**Table 1**) and comparable to control wounds and Integra in a two-stage procedure (**Table 2**).

Table 2. Take of the autologous skin in the two-stage procedure. *The percentage of the autologous skin that was still viable (take rate) at day 7 after application of acellular dermis or Integra is comparable to control wounds (directly closed).

	Number of wounds	Mean take rate*	Range take rate
Control	12	95	75-100
Glyaderm	8	90	60-100
Integra	6	96	55-100

Compared to the one-stage procedure, the number of inflammatory cells in the wounds treated with Glyaderm® was lower at day 7 and 14 after wounding. The influx of immune cells in Integra was comparable but some giant cells were present (**Figure 4A**). Eight weeks after injury, elastin fibers could still be observed in the Glyaderm®, surrounded by newly produced collagen fibers (**Figure 4B**) of which the majority was already replaced.

In the Glyaderm® matrix only a few accumulations of macrophages and lymphocytes could be observed, but around the Integra fibers some giant cells were observed 8 week post-surgery.

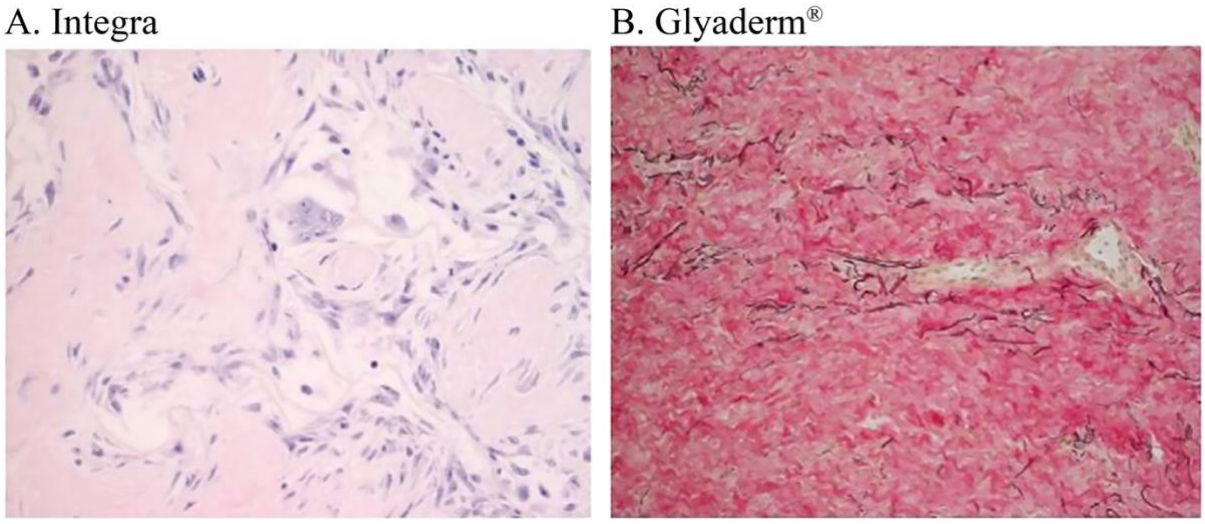

Figure 4. a) Presence of multi-nucleated giant cells with Integra. b) In the Glyaderm® treated site, donor elastin fibers are present after 8 weeks and newly produced collagen fibers are visible.

Effect of Glyaderm® in a two-stage procedure on contraction and scarring

Contraction of wounds treated with Glyaderm® in a two-stage procedure is reduced compared to Glyaderm® in one-stage procedure (**Figure 5**). Wounds treated with Integra showed the same contraction.

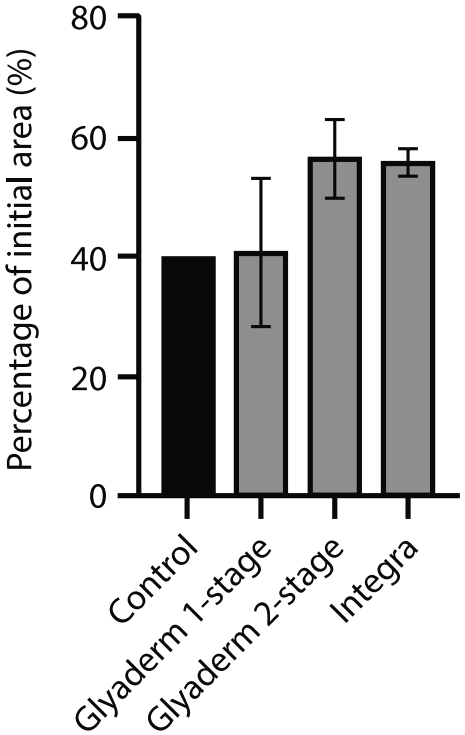

Figure 5. Wound contraction was measured at 8 weeks after the second operation (n=8 wounds).

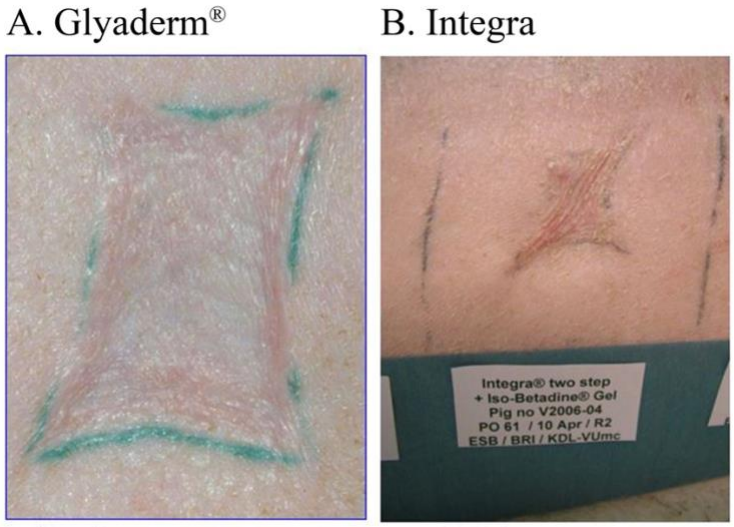

Figure 6. The scar quality of the wounds treated with Glyaderm® in the two-stage procedure seemed better compared to Integra, though not statistically significant.

Discussion

The aim of this study was to compare Glyaderm® with other human dermal matrices; Alloderm® and DED. The NaOH method to decellularize human donor skin (Glyaderm) is more thorough. Less cells and remnants of appendages could be detected in sections of the material. This could explain the milder inflammatory response in the wounds treated with Glyaderm® compared to wounds treated with Alloderm® or DED. As a consequence, wounds treated with Glyaderm® showed better results with respect to colour and smoothness of the scar. Nevertheless, this could only be observed in wounds with an adequate take rate.

Survival of STSG decreased when placed on a dermal matrix compared to the control wounds covered with only autologous STSG. The take rate on Glyaderm® was lower than on Alloderm® but higher compared to DED. The NaOH may have caused some damage to the basal membrane molecules which are important for outgrowth of the epithelial cells as has been shown *in vitro*[14] and *in vivo*[10]. The method for processing DED preserves intact basal membrane molecules[13] but the inflammatory response in the matrix may hamper the ingrowth of blood vessels from the wound bed. Although the inflammatory response was lower with Glyaderm®, no significant differences were observed with respect to wound contraction. The number of myofibroblasts, which are known to be related to contraction and scarring, did not differ[16-17]. For the initial survival during the first days, the autologous skin graft depends on diffusion of nutrients from the wound fluid before newly formed blood vessels are connected. It takes at least 4 days for the endothelial cells to invade the relatively tightly woven collagen and elastin fibers from the human skin derived matrices.

Thereafter, we tested Glyaderm® in a two-stage procedure with a one week interval between the dermal implantation and autografting. In our porcine model, this time period was sufficient to reach a take rate comparable to control wounds without Glyaderm® as well as wounds treated with Integra. The good survival of the STSG

indicated a fast ingrowth of fibroblasts and blood vessels into the Glyaderm® matrix, leading to reduced wound contraction compared to the control wounds. Interestingly, the numbers of inflammatory cells were also lower in wounds treated with Glyaderm® in the two-stage procedure compared to the one stage procedure, both early after wounding (day 7) and at day 56. In addition, the numbers of myofibroblasts in the scars at day 56 were significantly lower when compared to control wounds, covered with only autologous split skin. As expected, wounds treated with Integra also showed improved results compared to the controls. There were no significant differences in wound contraction and numbers of myofibroblasts between Integra or Glyaderm® in the two stage procedure, only the presence of giant cell formation in wounds treated with Integra was observed, potentially caused by glutaraldehyde crosslinking.

Druecke et al.[18] have described the use of Integra in a one stage procedure in a porcine model and did not observe differences in contraction between control and Integra-treated wounds. Thus, although two operations are needed, final results with respect to wound contraction and scar formation are much better if a dermal substitute like Integra or Glyaderm® is used in a two-stage procedure. Only thinner dermal substitutes with a more open structure that allow faster ingrowth of blood vessels may be used in a one-stage procedure[19]. These types of dermal substitutes lack the natural structures of collagen and elastin fibers present in Glyaderm® that can modulate fibroblasts to produce more randomly organized collagen fibers. Open pore structure matrices are more vulnerable to early degradation by matrix-metallo-proteinases (MMP's) produced by infiltrating fibroblasts and macrophages.

These good results obtained with Glyaderm® in the two-stage lead to a pilot study with a group of 12 burn patients[20]. Growth of blood vessels from the wound bed into Glyaderm® was assessed using laser Doppler imaging. An interval of 5-7 days between the first operation and the second operation was sufficient to achieve a take rate > 95 %. Thereafter, an intra-individual comparative clinical study was performed to evaluate the long-term effects. The elasticity of the scar was significantly improved at 1 year follow-up when using Glyaderm® [21]. Additionally, several layers of Glyaderm® could be applied on exposed bone and the wound could be successfully closed with STSG[22]. Biopsies taken 7 days after implantation clearly showed new collagen in the Glyaderm[23], confirming the observation in the porcine model. The human donor derived, native elastin fibers serve as a scaffold for the autologous fibroblasts, resulting in scar tissue with improved elasticity.

Conclusions

An acellular dermal substitute such as Glyaderm® can be successfully used to reduce wound contraction in the porcine wound model. Glyaderm® with a thickness > 0.5 mm should be used in a two-step procedure for optimal results. During the interval between the first and second step, blood vessels and fibroblasts will infiltrate the Glyaderm®. In this way, the survival of the STSG is improved, resulting in a better quality of the final scar.

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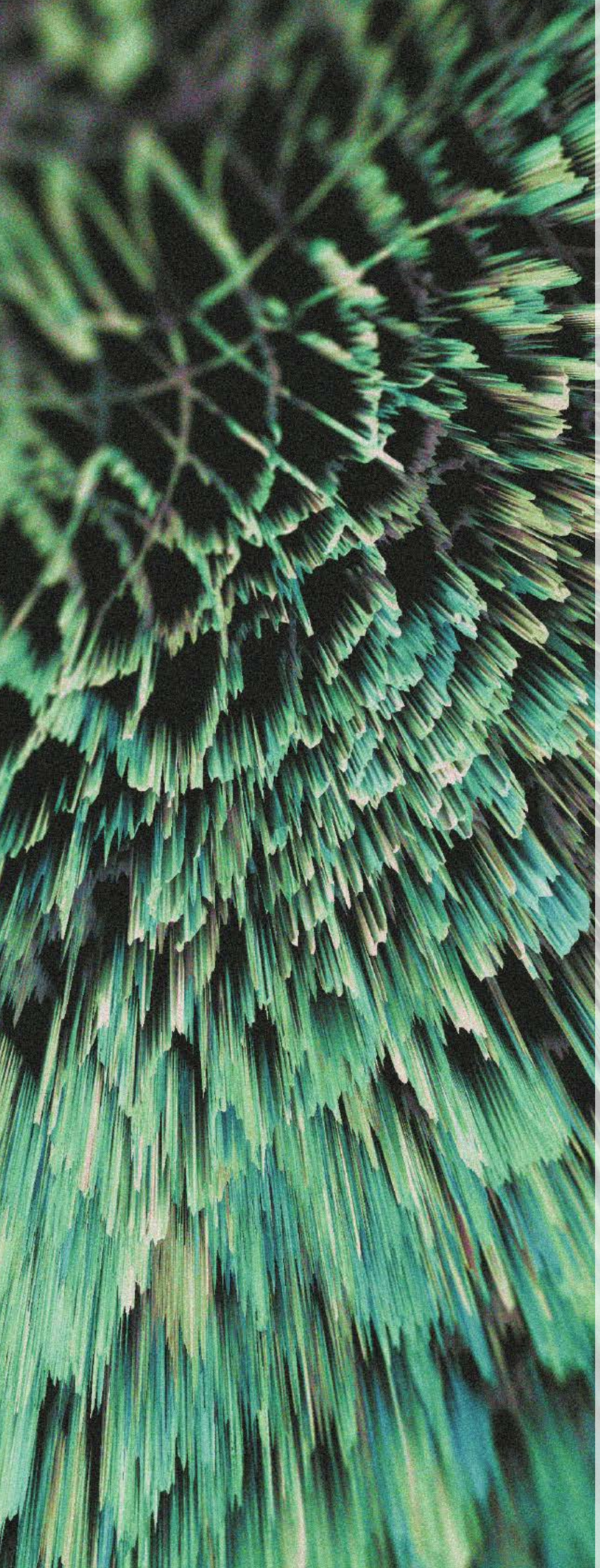

CHAPTER 4

EVALUATION OF ACELLULAR DERMIS FOR CLOSURE OF ABDOMINAL WALL DEFECTS IN A RAT MODEL

EUROPEAN SURGICAL RESEARCH 2008

A.PIRAYESH
A.H.M.DUR
N.J.PAAUW
S.MONSTREY
R.W.KREIS
M.J.HOEKSTRA
C.D. RICHTERS

Abstract

Background

Abdominal wall repair can be performed with synthetic or biological materials. Biological materials may reduce the risk of infections and fibrosis. The aim of this study was to evaluate two acellular human dermis products.

Materials and Methods

A rat model was used to compare the two materials. One was prepared using low concentrations of NaOH; the other material was SureDerm®, which is commercially available. Full thickness defects were prepared in the abdominal wall and closed with the materials. Rats were sacrificed at 1 or 4 months after operation and the numbers of adhesions to the bowels were scored. Samples were taken for histological analysis and to measure the breaking strength.

Results

In both groups a good functional integration of the implants with the abdominal wall was observed. There was no adhesion formation with the bowels in the group with the NaOH prototype. In the SureDerm® group, 4 out of 7 rats showed only small adhesions at 4 months after operation. Breaking strength of the healed tissue was significantly higher in the NaOH prototype group at 4 months after operation ($p<0.0026$).

Conclusions

The results indicate that both human acellular dermis products may be used in clinical trials for closure of abdominal wall defects.

Introduction

Abdominal wall defects resulting from trauma or incisional hernias are difficult to close for general surgeons and plastic surgeons. The prevalence is up to 10% of all laparotomies and these defects cause mechanical and physiological disturbances that increase patient morbidity. Small hernia repair by primary closure with suture has been associated with recurrence rates of up to 63% in a 7-year median follow-up. The introduction of mesh has resulted in a decrease of recurrence rates to approximately 30%, with most of the recurrences occurring within the 1st year after repair[1]. Various methods and materials for treatment are described, such as synthetic materials like polytetrafluorethylene (PTFE)[2] or polypropylene mesh[3]. The disadvantage of these materials can be the formation of fibrosis and abdominal adhesions[4]. In addition, a higher risk of infection is associated with synthetic materials.

In patients with comorbidity such as morbid obesity, diabetes and or a history of infection, complications such as mesh extrusion, infection and fistula formation have been reported ranging from 23 to 78%[5]. Plastic surgeons have developed techniques to allow hernia closure by autologous tissue mobilization without the use of prosthetic materials, which are prone to infection and fistula formation. These techniques involve medial mobilization of the abdominal rectus muscles either with or without separation of the components of the abdominal wall[6] or distant or free tissue transfer[7]. These techniques are also associated with recurrence rates of up to 30%.

The use of biological materials together with these techniques may reduce recurrence and associated comorbidity. Positive results are reported using human tissue such as dura mater[8], amniotic membrane[9], dermis or xenogeneic materials (porcine submucosa, bovine pericardium, porcine dermis)[10]. Recent studies showed favourable results using human acellular dermis in animal models as well as in patients (ADM[11] and Alloderm®[12,13]).

The aim of our study was to evaluate the effectiveness of two other types of human acellular dermis to close abdominal wall defects. Acellular dermis was prepared from human donor skin using two different methods to remove the donor cells and antigenic structures. One prototype developed by our research group is intended to ensure the integrity of the collagen elastin matrix and is reproducible without the use of expensive techniques and/or storage methods. The other acellular human dermis type is SureDerm®, which is commercially available (Hans Biomed, Seoul, Korea). Both types were tested as implants to repair full thickness defects in the abdominal wall of rats. The incidence of adhesion formation, inflammatory response and the tensile strength was compared at 1 and at 4 months after operation.

Materials and methods

Preparation of the implants

Donor dermal tissue was recovered with a dermatome from a donor (deceased person who gave informed consent) after the removal of the first layer of skin with the

epidermis. Thickness of this second layer of dermis was 0.8-1 mm. Donor dermal cells were removed using two different methods. With one method, the dermis was incubated in 0.06 N NaOH at room temperature for 6 weeks with shaking at regular intervals. The NaOH solution was replaced every week. After 6 weeks, the NaOH solution was neutralized using HCl and the tissue washed with 0.9% NaCl. This NaOH prototype acellular dermis was then stored in 85% glycerol until use in the rat experimental model. Processing in glycerol 85% of the material can be done without any effects on the structural integrity, and the glycerol has antibacterial and virucidal properties[14, 15].

The other material is produced according to a patent of Hans Biomed, the product SureDerm®, a human acellular dermis for soft tissue repair. The material is prepared using enzymes and EDTA. Thereafter the material is freeze-dried and can be stored at 4- 8°C. For the experiments, the type of SureDerm® for implant was used, with a thickness of 0.8-1 mm. This was obtained from Hans Biomed. Samples of both materials were taken for histology to assess the integrity of the collagen and elastin matrix. Haematoxylin and eosin and Elastica van Gieson staining were used.

Rat model

Twenty-eight rats were used (male Wistar, 180-200 g) obtained from Harlan-CPB (Zeist, The Netherlands). The local animal welfare committee of the VU Medical Centre approved the research protocol. The rats were kept under routine laboratory conditions with free access to food and water. They were kept in separate cages for 2 weeks after surgery.

Rats were anesthetized using a mixture of N2/O,/isoflurane. A full thickness defect down to peritoneal cavity in the abdominal wall of 1 by 1.5 cm was created, which was repaired using the NaOH prototype as implant in 14 of the animals. SureDerm® was used in the other group of 14 animals. Both materials were rinsed in 0.9% NaCl before use and then sutured with PDS I (Ethicon, Johnson & Johnson, Brussels, Belgium) to the defect with minimal tension. The skin was sutured with Vicryl 3/0 (Ethicon, Johnson & Johnson). Postoperative pain control was achieved with Temgesic (buprenorphine hydrochloride, 0.03 mg/kg rat) injected intramuscularly directly after the operation.

In both groups, 7 animals were sacrificed after 1 month, the other 7 animals at 4 months after operation. The abdominal cavity was opened carefully to score the presence of adhesions to the implants. Digital images were taken with a camera; these were used to estimate the size of the implants. Settings of the camera were the same for all animals. The animals were placed on a board of 15 by 25 cm and fixed at 4 points, always in the same way. The pictures were taken in such a way that the board was included to allow measurements. Thereafter, biopsies of the implants including abdominal wall without the skin were taken for histological analysis.

Biopsies for measuring breaking force were prepared only from the animals in the groups that were sacrificed at 4 months after operation. At this time point, the PDS sutures had been resorbed.

Immuno(histo)chemistry

Half of the samples were fixed in Kryofix and embedded in paraffin for routine haematoxylin-eosin staining. Haematoxylin and eosin were obtained from Gurr (BDH, Poole, UK). Sections were fixed in Baker's formalin before staining.

The other samples were frozen in liquid nitrogen. Cryosections were prepared to perform specific staining for rat macrophages using the ED-2 monoclonal antibody[16]. After fixation in acetone, slides were incubated with the primary antibody for 45 min at room temperature. Thereafter the slides were washed 3 times with PBS followed by incubation with a biotinylated secondary antibody (rabbit anti-mouse, Dako, Glostrup, Denmark) for 1h at room temperature. After washing with PBS, slides were incubated with alkaline phosphatase-conjugated streptavidin (Vector, Burlingame, Calif., USA). Positive staining was then visualized by using alkaline phosphatase substrate containing naphthol AS-BI phosphate (Sigma, St. Louis, USA) and new fuchsin (Gurr) resulting in red staining. 1mM levamisole (Sigma) was added to the substrate to block endogenous activity.

Two independent observers analysed the stained sections. The numbers of positive cells (ED-2-positive macrophages)/mm^2 of tissue were counted using a grid measuring $1mm^2$ (10 by 10 fields) in the ocular of the microscope. Five regions per section were counted, 2 were randomly selected on the border of the matrix implant, 3 in the center.

Tensile load testing

Tensile strength measurement was performed on a tensiometer (Instron 8872). Strips of tissue (width 1 cm) comprised the implant with 2 cm abdominal wall tissue without the skin. They were cut at both sides using a device with fixed knives. This device is a plastic block with 3 blades placed at a distance of 1 cm. By using this device, strips of tissue of the same size could be obtained. Stretching velocity was 2 cm/min.

Statistical analysis

The $\chi 2$ test was used to analyse the adhesion formation data. The unpaired t test (two-tailed) was used to compare the results of the number of macrophages in the implants and the tensile strength.

Results

Adhesion formation

Animals were checked daily for local or systemic complications. None of the rats suffered from infection or other complications related to the implants. They were euthanized at 1 and 4 months after the operation, and the presence of adhesions was scored. All rats had mild adhesion of the omentum to the borders of the implants. The group with the NaOH prototype implant had no adhesions of the intestines whereas in the group with the SureDerm® implant, 4 out of 7 (after 1 month) or 3 out of 7 rats (after 4 months) showed adhesion of the small bowel to the implant (**Table 1**). Although this is a significant ($p = 0.018$ and 0.051) difference, these adhesions were only mild adhesions, as shown in **Figure 1**, which were limited to one area. In addition, we observed in some rats adhesions of the implant to the skin, especially in the group with the NaOH prototype implant (3 out of 7 rats at 1 month, $p = 0.051$). None of the rats in the group with this implant showed adhesion to the skin at 4 months after operation and only 1 rat of the SureDerm® group (not significant, $p = 0.299$). No signs of seroma adhesion could be observed in the rats with adhesions to the skin.

		NaOH prototype	SureDerm®	p value
1 month	Bowel	0	4	0.018
	Skin	3	0	0.015
4 months	Bowel	0	3	0.051
	Skin	0	1	0.018

Table 1. Number of rats showing adhesions (n = 7 in each group)

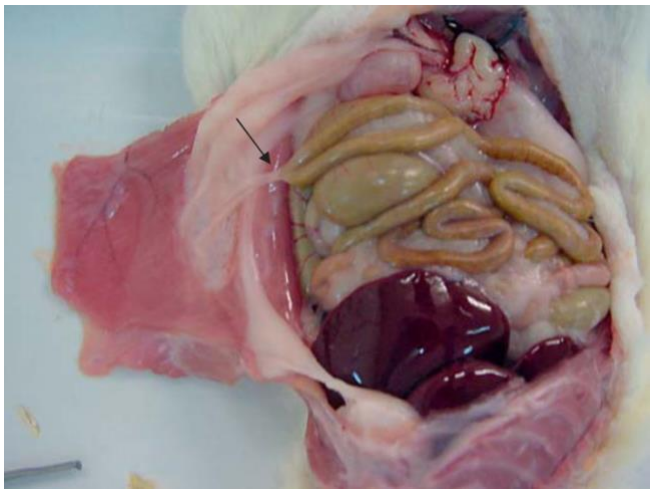

Figure 1. Example of mild adhesion formation of the small bowel to the SureDerm® implant 4 months after operation. The omentum also shows some adhesion to the implant.

Histopathology results

The sections of both materials before implantation showed intact collagen and elastin fibers. Although treatment with NaOH is a stringent method, the low concentration used to obtain the prototype did not result in damage to the matrix as shown earlier[17]. Good integration of the implant with the abdominal wall was observed in all rats, at 1 month after operation as well as in rats 4 months after operation. A fibrous collagen layer was present, connecting the implant with the wall (**Figure 2**). This layer covered the implants; some blood vessels were growing from the layer into the implant. The human-derived collagen of the implants could be easily distinguished from new rat collagen; the implant fibers are thicker and larger.

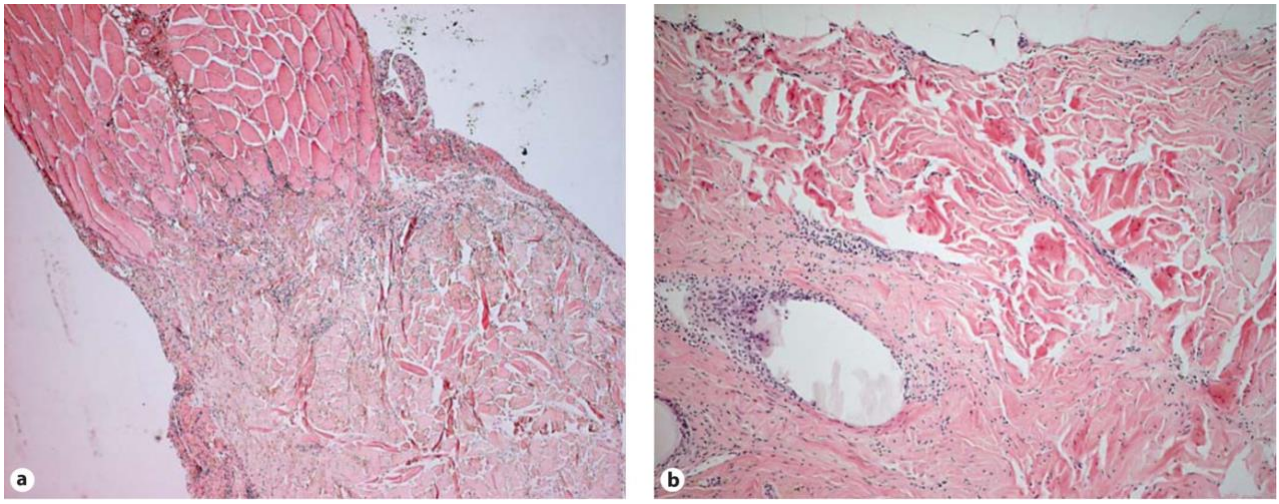

Figure 2. HE-stained sections of the implants. A strong connection between abdominal wall and implant was formed both in the group treated with the NaOH prototype (a) as well as in the SureDerm®group (b) 4 months after surgery.

In none of the rats, relatively short fibers could be observed indicating that new collagen deposition in the implants had not taken place. The implant-derived collagen was still *in situ* after 4 months in the NaOH prototype group but there were no signs of replacement by host collagen. No clear signs of contraction of the implants could be observed. The implant was partly absorbed (as estimated from the digital pictures, up to 40% reduction of the original thickness) in 4 out of 7 rats (range 0-40%) in the SureDerm®group.

Inflammatory cells (mostly mononuclear cells) were observed around sutures but also in the implant. More cells were observed in the SureDerm® implants compared to the NaOH implants (**Figure 3**). Specific staining for macrophages (ED-2) showed that these cells were present in the implants (**Figure 4**); the number of cells was significantly higher in the SureDerm® implants (**Figure 5**).

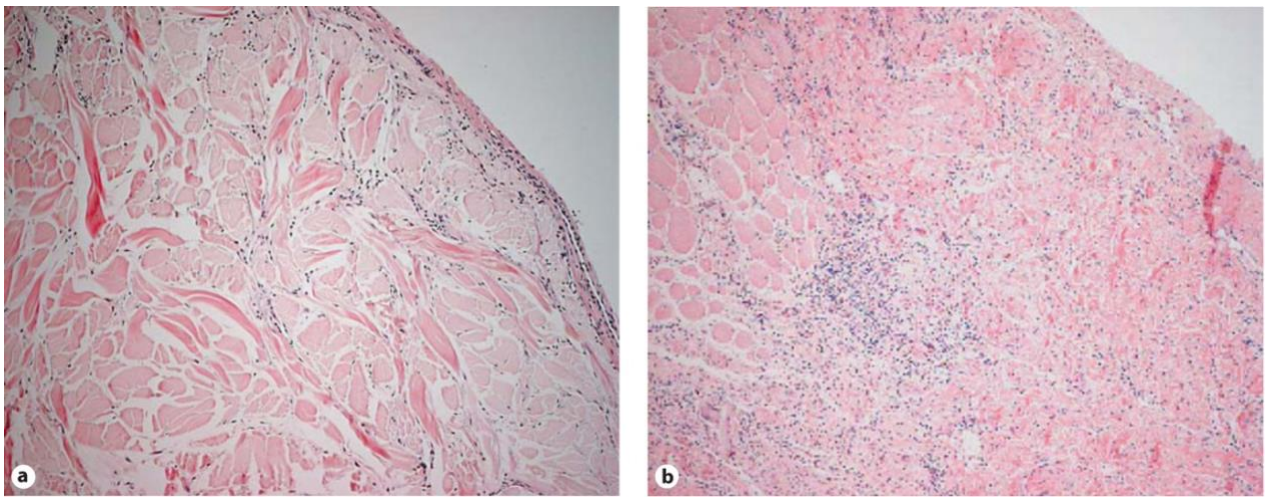

Figure 3. HE-stained sections of the implants 4 months after surgery. More inflammatory cells are present in SureDerm®(b) compared to the NaOH prototype (a).

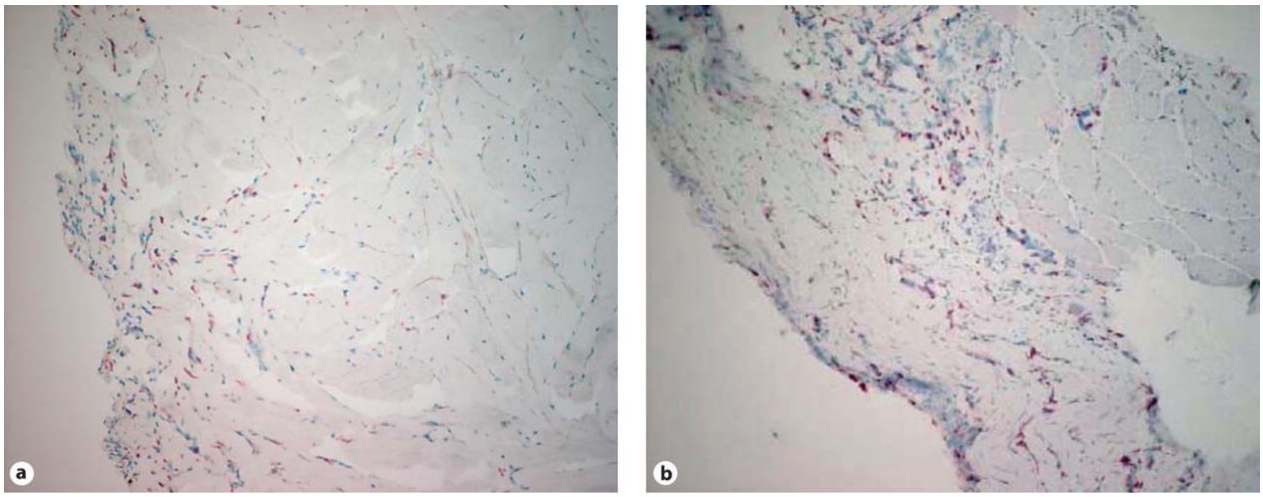

Figure 4. Sections of the implants stained with the ED-2 antibody, recognizing rat macrophages (red cells). More cells are present in SureDerm®(b) compared to the NaOH prototype (a) 4 months after surgery.

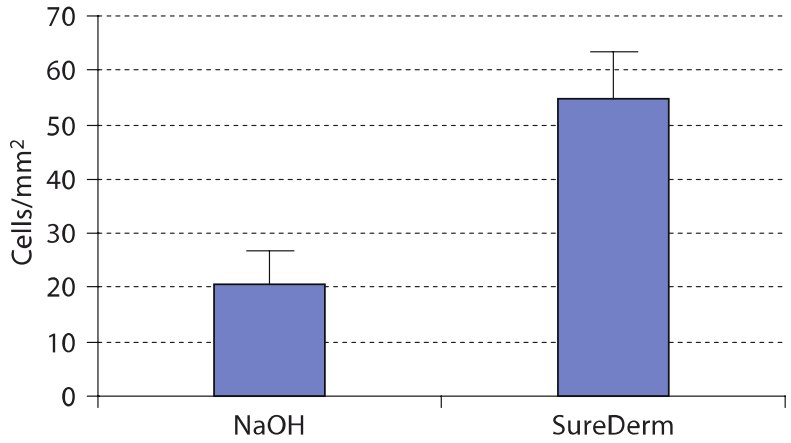

Figure 5. The number of ED-2-positive macrophages is higher in the SureDerm® implants 4 months after operation (mean 54.8 + 8.5) compared to the NaOH implants (mean 20.5 + 6.1). There is a significant difference ($p < 0.0001$). Data are expressed as the mean number of cells/mm? (+SD) of 7 rats for each type of implant.

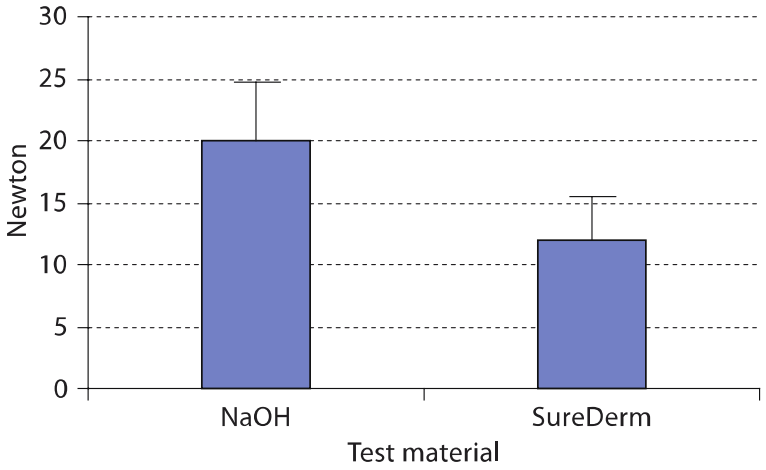

Figure 6. The breaking strength of the healed tissue is higher in rats treated with the NaOH prototype implants (mean 20 + 4.7) compared to rats with the SureDerm® implants (mean 12 + 3.5) 4 months after surgery. There isa significant difference ($p<0.0026$). Data shown are mean + SD with 7 animals in each group.

Blood vessels and fibroblasts were also present in the implants; higher numbers (up to 2-3 times higher) were observed in the SureDerm® implants. In the NaOH implants, some calcification was observed around the area with sutures.

Tensile strength

At 4 months after the operation, the NaOH implant showed a higher tensile strength compared to the SureDerm® implant (**Figure 6**); this was a statistically significant difference ($p < 0.0026$, unpaired t test). The strips of tissue tore at the border of the implants in the fibrous tissue between the implant and the tissue.

Discussion

Incisional hernia is a common problem encountered by surgeons in numerous subspecialties. Repairs that use mesh prosthesis are associated with lower recurrence rates.

The use of mesh material with more favourable properties than traditional mesh in abdominal wall reconstruction could have a positive impact on surgical practice and patient outcomes. The ideal biomaterial would resist infection immediately after implantation, become completely remodelled into host tissue with mechanical and biological properties identical to those of the missing tissue, become rapidly revascularized and infiltrated with cells, resist seroma formation, and maintain its original strength and surface area during remodelling to prevent bulge, failure, or stretch. This ideal material has yet to be discovered or produced. Fortunately, certain

bioprosthetic mesh materials have properties that may make them closer to this ideal than traditional meshes.

In the present study, we have compared two prototypes of biomaterials to close abdominal wall defects in a rat model. These biomaterials were derived from human donor skin by different methods, an NaOH-treated prototype implant and a commercially available dermis (SureDerm). Both types of human donor skin-derived implants can be successfully used in the rat model. There was a good integration of the implant with the abdominal wall at 4 months after operation. None of the rats had complications such as infection or incisional hernia. Only mild adhesions to the omentum were formed in all rats and in some of the rats in the SureDerm® group, mild adhesions to the small bowel were observed. The NaOH prototype induced adhesions to the skin in some of the rats at 1 month after operation but at 4 months only 1 rat of the SureDerm® group showed adhesion to the skin. These are favourable results if compared to results in rats using 2 X 3 polypropylene mesh or PTFE with polypropylene mesh. Demir et al.[18] reported thick adhesions involving more than 50% of the material in most of the rats. We observed some adhesions of the materials to the skin without a clear explanation; this is still under investigation.

It has already been shown in animal models that Alloderm® can be successfully used for abdominal wall reconstructions[13]. It can be safely used in patients for abdominal wall repair[12], but the material is expensive. In this respect, Schuster et al.[19] advised to use Alloderm® only if wound closure with skin can be achieved during operation. Alloderm® is an acellular dermal matrix derived from human donor skin[20]. It is prepared using a patented procedure by LifeCell. The results of our rat study indicate that other types of acellular dermal matrix obtained by different methods may be used with the same optimal functional results.

Incubation of human skin with low concentrations of NaOH is a simple and cost-effective method to remove donor cells and hairs. A prototype derived from the upper dermal layer of the skin with this method[17, 21] can also be used to improve scar quality and to reduce contraction in burn wounds when placed underneath an autologous split skin graft and is currently undergoing phase III clinical assessment. The method to obtain SureDerm® is a patented procedure of Hans Biomed and the product can be ordered for clinical use, for instance burns. So far there have been no reports of the use of this material in abdominal wall repair. Although the functional results are good for both materials in our rat model, we observed some differences. In the SureDerm® implants, more of the material was resorbed at 4 months after operation; this was observed in 4 out of 7 rats. Most probably, this is due to the significantly higher number of inflammatory cells (predominantly macrophages) that were present in the SureDerm® implants. The NaOH method may be more stringent compared to the method to obtain SureDerm, leaving fewer antigenic structures in the implants that can induce an inflammatory response. The lower breaking strength of SureDerm® is most likely the result of this resorption of the material by macrophages.

We observed that on both materials, a well-organized collagen layer was formed, connecting the abdominal wall with the implant and as a parallel layer on the implant covering it. This resulted in a functional closure of the defects without complications

in most of the rats indicating that the materials are suitable for use in patients. We did not observe any replacement of the collagen of the implant by new collagen produced by host fibroblast infiltrating the implant. This is in agreement with the results of Zheng et al.[10]; they described in a study on porcine dermal collagen in rats that the implant also became encapsulated rather than replaced by new tissue. This may be due to the xenogeneic origin of the implants. The human skin is made acellular and induces only a limited inflammatory response. The structures of the extracellular matrix molecules may differ from rat molecules in such way that collagen production is not induced in the rat fibroblasts. In future experiments, we will study the materials in large animals such as pigs and in an allogeneic setting, i.e. porcine skin treated with NaOH or with the SureDerm® method before proceeding with a clinical study.

Bioprosthetic materials have increasingly become the subject of clinical and scientific interest, and their clinical use for abdominal wall reconstruction has increased over the last years with impressive results, particularly in adverse situations. However, a considerable amount of information has not yet been elucidated or quantified with respect to the biological and physiological mechanism of action and long-term outcomes of abdominal wall reconstruction with bioprosthetic materials. The indications and contraindications for their use have not been defined clearly and likely will become clearer with continued basic science studies and clinical outcome data. Additional evidence-based study data will allow modifications of existing materials and introduction of new products with superior properties and function, which will enhance management of abdominal wall defects.

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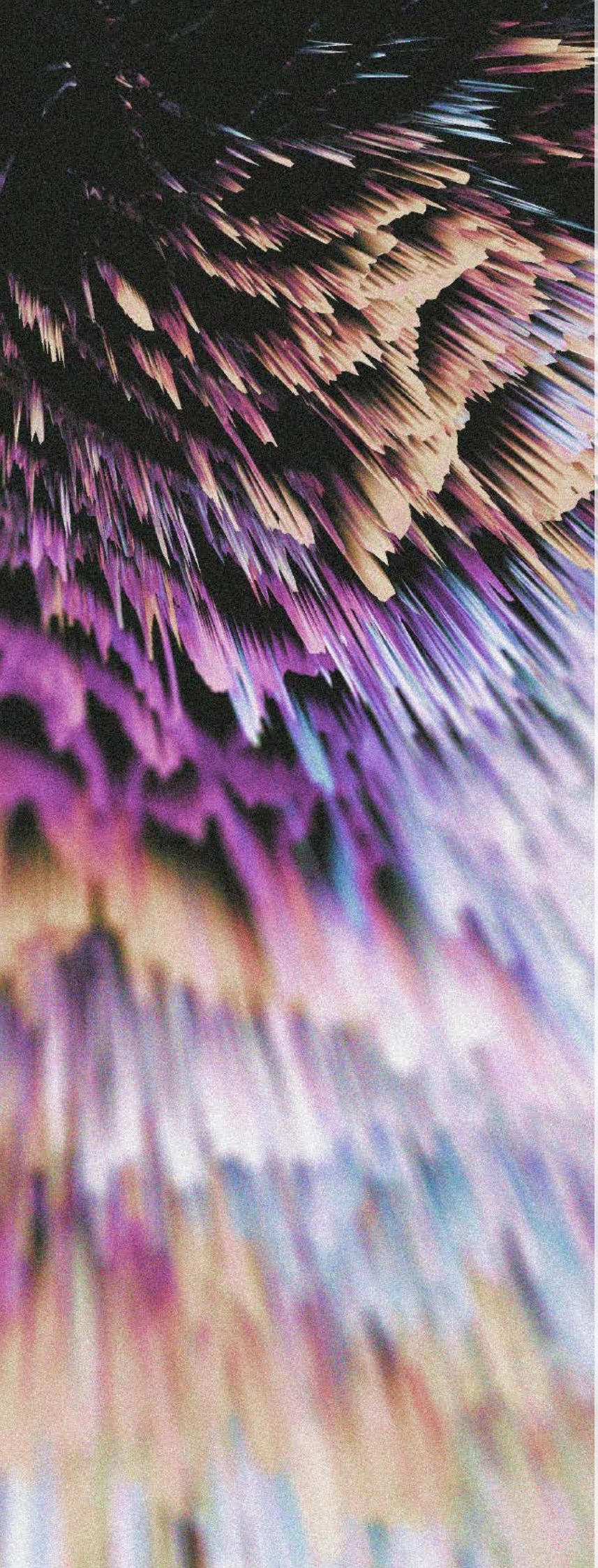

CHAPTER 5

SKIN REPLACEMENT IN BURN WOUNDS

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N. BRUSSELAER
A. PIRAYESH
H. HOEKSEMA
C.D. RICHTERS
J. VERBELEN
H. BEELE
S.I. BLOT
S. MONSTREY

Introduction

Historically, the main goal in burn management was increasing the survival of severely burned patients by rapid debridement and early closure of burn wounds, consequently reducing the infection risk[1-4]. However, in the last decennia, surgical emphasis has shifted from survival to "quality of survival," especially by improving the residual scars and preventing contractures. Traditionally, surgeons divide burns into deep burns requiring surgical therapy, and superficial burns which heal spontaneous by re-epithelialization with minimal scarring. Nevertheless, there is a grey zone between those two groups in which therapeutic decision making is difficult. The final decision for surgery generally remains case and surgeon dependent, and will mainly depend on the total burned surface area[5]. Wound closure can be obtained by diverse therapeutic modalities depending on the depth and healing potential of the burn wound[5]. In this article, the main focus is on the surgical treatment of deep dermal and full thickness burns. We endeavour to give a comprehensive overview of the developments in skin substitutes, which is impossible without mentioning some alternative treatments.

The current golden standard for deep burns is surgical debridement and closure with autologous split thickness skin grafts or "STG" (epidermis plus a thin layer of dermis). Nevertheless, donor areas are limited in extended burns, and the residual scars remain unsatisfactory due to the lack of dermis. A more aesthetical reconstruction can be obtained with full thickness skin grafts (epidermis and whole dermis), which are limited in dimension and can only be harvested in a few areas (groin, lower abdomen, etc.). Deep defects with exposed bone or neurovascular structures are currently treated with flap surgery, which gives an optimal aesthetical and functional result. Nevertheless, the severe donor-site morbidity, the technical difficulty, and sometimes severe complications limit its use mostly to secondary reconstructions. Consequently, alternative conservative and surgical treatments were developed to improve the healing and the quality of the residual scars[6]. Several mechanisms are supposed to enhance healing:

(i) providing the ideal wound environment (wound dressings, etc.)
(ii) by assisting the intrinsic healing capacities (growth factors, cytokines, etc.)
(iii) by surgically replacing the damaged skin ("skin substitutes"), which also should reduce scarring in full thickness defects.

A permanent skin substitute is a surgically fixated "long lasting" skin replacement, consisting of naturally occurring skin elements which become incorporated in the normal skin. The main issue of this definition is the longevity of a skin substitute, which seems to be mostly of commercial importance, where terms such as biological dressings, and permanent and temporary skin substitutes are used without a clear distinction. This literature review showed that technically similar products are commercialized as "permanent" by one company and as biological dressing by another. Therefore, we chose to divide all these products in the following categories, depending on the skin layer which is (temporary) replaced: epidermal, dermal and combined skin substitutes (or composite grafts; **Figure 1**). In the future, a fourth

group might need to be added: the combined skin substitute with a subcutaneous adipose layer[7]. However, the difference between skin replacements and some wound dressings can be small. Wound dressings are intended for coverage instead of replacement, to optimize wound healing. Wound dressings can roughly be divided in dressings containing natural elements (such as honey ointments), synthetic dressings (such as silver-impregnated dressings), and biological dressings containing mammalian cells or cell-derived substances like collagens and growth factors (human donor skin). Synthetically manufactured, naturally occurring elements, such as cellulose membranes, are also synthetic dressings. Wound dressings are not considered as (permanent) skin substitutes because they are not incorporated in the healing wound. Some authors previously named some of these products "skin substitutes" (without mentioning "permanent") but this only lead to confusing terminology.

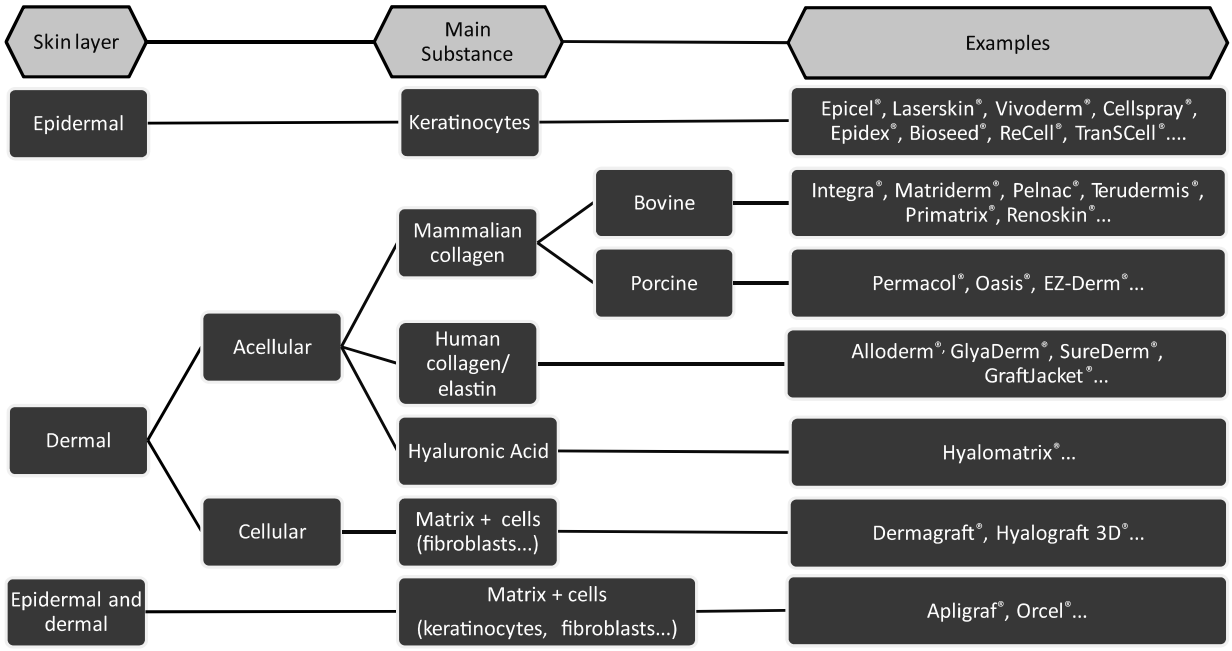

Figure 1. Classification of skin replacements.

The most important biological dressing, used since the 1940s, is human donor skin or "cadaver skin"[8]. It contains several beneficial factors (growth factors, cytokines, etc.), and it provides the ideal environment for healing. Because of better preservation techniques (glycerol or cryopreserved), the risk of infection transmission is minimized, and its rejection will be delayed up to 3 weeks to 5 weeks[8,9]. One of the currently most popular temporary dressings, is Biobrane, a nylon-collagen mesh, often used for partial thickness burns[10].

The skin substitutes can consist of several elements, depending on the skin layers which need to be replaced (**Figure 1**). The epidermal substitutes consist mainly of keratinocytes, dermal substitutes of major extracellular elements (collagen, elastin,

adhesion glycoproteins, and/or hyaluronic acid[11]), and sometimes also fibroblasts. Combined skin substitutes or composite grafts contain at least keratinocytes and a dermal matrix. Some authors prefer to use "bilayered skin substitutes" instead, but this terminology is confusing because some silicone-covered dermal substitutes are also labelled as "bilayered." If skin substitutes contain allogenic cells (from neonatal foreskin, etc.), those will not be incorporated in the healing skin (because of the immunogenic rejection), but they do stimulate healing by secretion of cytokines and growth factors. When allogenic cells are combined with a dermal matrix which becomes incorporated, we consider them nevertheless as permanent skin substitutes.

The aim of this study was to give an overview of which types of skin replacements have been developed and which problems still need to be faced. None of these commercialized products can currently claim to be the optimal skin replacement, because clinical evidence is too scarce (several large multicenter trials are currently in process). The number of products becoming commercialized is nevertheless increasing steadily, which pleads for a certain overview, classification, and clear comparison of the available products.

Epidermal skin substitutes

The currently most popular method for epidermal restoration is with STG, which was introduced clinically in 1823[12]. Because of limited donor area in extended burns, other epidermal replacement techniques were examined.

The first attempt to accelerate wound healing with living cells dates from 1870. Healthy skin (containing keratinocytes) was scraped off and applied to the wound bed. The best results were obtained when deeper parts of the skin were used[12]. However, a good technique for culturing keratinocytes was only obtained in 1975[13].

To obtain a large amount of autologous keratinocytes, a biopsy (25 cm^2) is taken from healthy skin and cultured during 2 weeks to 3 weeks on a nutritional layer[14,15]. Specialized private or hospital-based laboratories developed several preparation and application techniques - gels, sheets, or sprays - such as Epicel, Laserskin (Vivoderm) (on a hyaluronic acid scaffold), Cellspray, Epidex (keratinocytes derived from hair roots), Bioseed-S, ReCell, and TranCell[14-21].

Allogenic keratinocytes (and fibroblasts) can be obtained from fresh human donor skin, neonatal foreskin, and surgical resections (such as abdominoplasties and breast reductions). "Chimerical" keratinocyte cultures, composed of allogenic and autologous cells, can be applied in ratios like 20:1 (less autologous cells needed)[22,23]. but clinical studies are scarce. Histopathologic follow-up showed only autologous cells in the regenerated epidermis after 1 month to 2 months[22,24]. There were already attempts to combine the keratinocytes with melanocytes and Langerhans cells to create a more complete epidermal substitute, but the clinical significance remains to be determined, but might be very useful in the treatment of vitiligo[25].

The main disadvantages of keratinocyte cultures are the variable take rate, the high susceptibility for infections, the long cultivation time, and the high costs[26-28]. After

healing, the skin remains fragile with easy blistering for up to 6 months to 12 months, due to the lack of dermis and a dermoepidermic junction[29]. A higher need for reconstructive surgery is also reported for the release of contractures[27,30]. Therefore, the use is limited to severe burns with limited donor area, for the donor area (usually autologous cells) and chronic wounds (usually allogenic cells)[14,15]. They are often combined with widely meshed STG or human donor skin[31,32], and in the future probably with dermal substitutes.

Replacement of the dermal layer

For full thickness skin defects (also severe damage of the dermis), application of epidermal cells is insufficient. Full thickness skin grafts (epidermis + dermis) can be used for small full thickness defects and give better results than epidermal replacement techniques, but donor areas are even more limited (groins, behind the ear, and lower abdomen). The dermis consists mainly of connective tissue (collagen, elastin, hyaluronic acid, etc.) produced by fibroblasts[33], contributing to strength and elasticity of the skin. The lack of dermis results in severe contraction and hypertrophic scarring especially in regions around joints[6]. Already in the early phases of wound healing, a number of fibroblasts will differentiate into myofibroblasts[34,35], but the keratinocytes themselves are also responsible for contraction, even in absence of fibroblasts[36]. After application of a dermal layer, the epidermal layer can be reconstructed by adding a (meshed) STG or cultured keratinocytes[37].

Nowadays, several dermal substitutes are used clinically (**Table 1,2**). They can be divided in two large groups: acellular substitutes and cellular substitutes which include living cells (fibroblasts and endothelial cells; **Figure 1**).38 The 3D matrices should enable progressive vascularization and invasion of fibroblasts from the surrounding tissues[39 – 41]. This should result in a mix of the foreign matrix and "native" material, histologically similar to normal skin. The fibroblasts will synthesize extracellular matrix components, cytokines, etc., which will eventually replace the skin substitute completely after several weeks, months, or even years, depending on the longevity of the material. The cellular skin substitutes are also metabolically active because they contain and synthesize cytokines, which improve healing.

Preventing rejection of human donor skin

The first attempts to reconstruct the dermis were by preventing rejection of human donor skin. Temporary treatment with immunosuppressive drugs like cyclosporine prevented the rejection even permanently[42-44], but this method was abandoned because of the side effects.

Based on the knowledge that rejection was mainly caused by the more immunogenic potential of the epidermal cells, Cuono et al.[16,17] proposed to remove the epidermis by abrasion, several days after grafting human donor skin. The dermis of the human

donor skin becomes incorporated into the recipient and provides a dermal bed on which cultured epidermal sheets can be placed[16,17,45]. However, technical difficulties in consequently removing the epidermal layer and immunologic reactions limit the use.

A third method is the intermingled technique: small pieces of autograft are placed in the interstices of a widely meshed human donor skin. This provides immediate coverage with the human donor skin and allows the autograft epidermis to slowly replace the allograft as rejection proceeds[46].

Development of the first dermal scaffolds

In the meantime, the first dermal scaffolds were developed, which could be used as such or as scaffold for combined skin substitutes. The initial attempts established to make multi-layered keratinocyte cultures easier to handle, by adding collagen hydrated gels or lattices[47-51]. Severe shrinkage during culturing restricted the use, which was partially prevented by anchoring methods *in vitro*[52]. An epidermal layer (STG or keratinocyte cultures) can be placed on top of the applied dermal matrix, immediately or after vascularization of the dermal matrix (1-3 weeks later)[37]. This "two-step procedure" can be indicated if the donor sites are limited (time to heal before reharvesting) but by postponing full closure, the risk of infection can be increased. The one-step procedure is limited by the slow vascularization of the dermal substitutes, which disables oxygen and nutritional transport to the epidermal layer[53]. The vascularization is very important to form a dermoepidermic junction, necessary for epidermal survival.

Initially, synthetic scaffold materials such as poly(L)- lactic acid and polyglycolic acid were examined[54]. These products have predictable and reproducible mechanical and physical properties (tensile strength, pore size, etc.) and can be manufactured with great precision. However, synthetic materials tend to elicit a foreign material type of response, specifically, a fibrous connective tissue deposition leading to formation of dense scars and fibrosis[54]. Therefore, naturally occurring materials such as hyaluronic acid and purified collagen have been investigated as alternatives to synthetic scaffolds. Collagens provide a unique combination of strength and flexibility, and they are the largest single component in the extracellular matrix and have a low antigenicity. Consequently, collagen is the most popular molecule for dermal scaffolds. The first stable collagen matrix was developed by Bell in 1979[55] which lead to the development of Integra (cf. infra). Native collagen (from human, bovine, or porcine origin) seemed to be superior to synthetically reconstituted collagen, because native collagen degrades less rapidly[41,56,57], but allergic reactions may occur. Reconstituted collagen degrades within 7 days, whereas a native collagen matrix remained detectable almost 6 weeks. Cross-linking improves the survival of collagen and other molecules, leading to an increased tissue half-life and better tensile strength, but it increases rigidity and reduces cellular affinity[54,58].

Another important and very stable dermal element is elastin (half-life 70 years), which provides strength and elasticity to the extracellular matrix[59,60]. Elastin is most

useful in skin replacements when organized in its naturally occurring network. When purified, the matrix can be severely damaged or elastin fibers can be separated, leading to accelerated degradation.

A smaller part of the extracellular matrix consists of hyaluronic acid, which has the significant advantage of structural conservation regardless of the source. Therefore, it does not cause allergic reactions when purified. It has been used therapeutically since 1968[61,62] and is nowadays available as meshed sheet for wound healing and as viscous hyaluronic gel for instillation into cavities (joint pathologies and eye surgery)[62]. Hyaluronic acid and collagen are also used widely in cosmetic surgery as dermal soft tissue fillers for softening of wrinkles and or volume restoration[63].

Currently available acellular dermal matrices

This group of dermal matrices contains all products mainly based on collagen or hyaluronic acid (**Table 1,2**). The collagen can be not only obtained from bovine or porcine dermis or tendons but also by processing human donor skin. Collagen can be extracted and remodelled as a 3D scaffold, or the original collagen skeleton can be conserved through a decellularization process. Previously, bovine collagen was more popular then porcine collagen, especially in wound treatment and skin reconstruction (probably due to a higher availability of cattle). Nevertheless, porcine collagen is regaining interest (also in cosmetic surgery), with as big advantage the absence of Prion diseases[64]. Other porcine viruses also needed to be considered, and porcine collagen might elicit more foreign body reactions than bovine collagen[65]. In addition, the literature on porcine dermal matrices remains scarce. Finally, religious and cultural differences need to be considered when using porcine and bovine tissues.

Bovine collagen

The main representative of this category, Integra, has been developed by Yannas and Burke[39,40,66 – 68]. It is approved for use in burn injuries since 1997 and is currently the most commonly used skin substitute in burn care and reconstructive surgery[69]. This acellular dermal substitute, also named “artificial dermis,” is made of a bovine collagen matrix (Achilles tendon) and glycosaminoglycans of shark cartilage, with a silicone layer on top to prevent dehydration and infection, although the use of antibiotics is advised[39,40,66,67,70 -72]. Because of the slow vascularization of Integra, the epidermal layer will be applied after 2 weeks to 3 weeks to obtain an optimal take. Recently, Integra Single Layer (without the silicone layer) became commercialized, which enables a one-step procedure[73]. The vascularization could be accelerated by applying negative pressure therapy[74]. This one-layer version can also be applied underneath the original Integra to treat full thickness defects. A similar matrix (without the silicone layer) is Duragen, also produced by Integra Life Sciences Inc. and used for neurosurgical interventions[75]. Five weeks after Integra grafting, the implanted products are biodegraded and replaced by their endogenous analogues[54].

Pelnac®[58,72,76 - 84] and Terudermis®[85- 87] are Japanese artificial dermal matrices based on the same principles, but consist only of "atelocollagen," covered with a silicone sheet. Atelocollagen is a highly purified trypsin-treated collagen I derived from calf dermis. Suzuki et al.[39,40,78] found no significant improvement when glycosaminoglycans was added, and therefore they commercialized Pelnac® without, leading to lower manufacturing costs. The difference between both products is the cross-linking method. Terudermis® is thermally degraded and cross-linked, which may be favourable for cellular affinity[87]. Pelnac® is chemically cross linked, which would produce a more durable result (higher resistance against the collagenases produced by the fibroblasts)[58,80].

A French dermal matrix is Renoskin®, which is composed of a reinforced silicone film and a porous matrix made from pure cross-linked bovine collagen[88]. Another non-cross linked product in this category is Primatrix®, which is based on collagen from fetal bovine dermis. Fetal tissues have been shown to have exceptional regenerative capacity and have a reduced transmissible spongiform encephalopathy infectivity (Creutzfeldt-Jakob disease, etc.), which is also the fact for adult skin[64]. The first scientific results remain to be published.

In animal studies, the addition of elastin to the collagen matrix resulted in a reduced cellular influx, a decreased number of myofibroblasts and more randomly orientated collagen bundles resembling normal skin[57,89]. This matrix is commercialized as Matriderm® and can be used in a one-step procedure[90-92]. It consists of collagen (bovine dermis) coated with elastin hydrolysate from the ligamentum nuchae. The first clinical results seem to be promising[91,92]. In burn wounds, MatriDerm® seems to degrade sooner than in reconstructive wounds[93]; and, after 3 months, results are comparable with the standard STG treatment, with no statistical evidence of long-term clinical effectiveness after 1 year[90].

Porcine collagen

The information about biological materials derived from pigs remain scarce. Some *in vitro* and *in vivo* trials are published already[94,95], but as far as we know, only Permacol is commercialized as dermal matrix for skin regeneration[96-98]. Permacol can be applied as dermal substitute but can also be used as combined skin substitute (with cells), but clinical results remain to be published[96]. In hand surgery, Permacol was studied as interposition graft after trapezoidectomy, but this study was discontinued because of severe tissue reactions[65]. Some porcine products are currently marketed as biological wound dressings (Oasis, E-Z-Derm®), but they are thought to act as dermal matrices[99]. E-Z-Derm® is composed of crosslinked porcine collagen, and Oasis is derived from porcine small intestinal submucosa, which seems to serve as a reservoir for cytokines and cell adhesion molecules, providing a scaffold for tissue growth[100]. The structure and biochemical composition of small intestinal mucosa supports tissue-specific remodelling, and the first clinical results were promising for partial thickness chronic and acute wounds.

Human donor skin

Human acellular dermal matrices (ADM) are derived from human dermis (**Table 1**), treated to remove all immunogenic elements: keratinocytes (also present on sweat and sebaceous glands), fibroblasts, vascular endothelium, and smooth muscle. Virus screening is also obliged. However, several different methods for processing those matrices have been developed[101-104], all aiming to preserve the integrity of the remaining dermal elements as good as possible. The main elements of all ADM are the collagen and elastin fibers, which serve as a 3D natural matrix for the invasion of the native cellular elements *in vivo*. The amount of remaining growth factors, cytokines, etc., depends on the processing method. The first ADM were processed by trypsin[101,105,106], freeze-thawing[102,104,107-109], or long incubations with enzymes[103,110]. Most of those matrices remained highly antigenic, which lead to poor graft survival[101-106,111,112]. At least five different manufacturing processes are currently registered for wound care. Some other techniques (like freeze- thawing) are still used for the processing of combined skin substitutes, but as far as we know, not commercialized as dermal substitute.

Alloderm® is a freeze-dried cryopreserved acellular dermal matrix on an intact basement membrane complex obtained by processing human donor skin in a saline solution (sodium dodecyl sulfate) and enzymes[37,53,112-117]. It is decellularized, freeze-dried, and biochemically stabilized, and has been successful alone and in combination with cultured autografts (two-steps procedure) in the treatment of burn wounds and dermal defects[37]. Additionally, Alloderm® is procured by cryopreservation which may affect the integrity of the elastin matrix, and its manufacturing is expensive.

DermaMatrix[118,119] is human donor skin processed using a combination of detergent and acid washes and is then freeze dried. It is especially commercialized for reconstructive surgery, but clinical studies in wound care remain to be published.

Glyaderm® is another acellular dermal collagen-elastin matrix, obtained by the treatment of glycerolized human donor skin with a low concentration of NaOH. The elastin matrix is not damaged by this manufacturing and preservation method, which should lead to a more durable effect[120,121]. Additional advantages of glycerol preservation include inactivation of viruses and ease of storage and handling[8,9]. Glyaderm® is provided by a non-governmental, non-profit organization, the Euro Skin Bank (the Netherlands) and is intended to be cost-effective, enabling widespread application. Glyaderm® is most effectively applied in a two-step procedure within a 6- to 8-day interval between Glyaderm® application and thin split thickness skin graft engrafting. Initial clinical studies are promising, with randomized and multicenter trials underway.

GraftJacket® is an acellular human dermis commercialized for deep, chronic diabetic foot ulcers[122-124]. Because these wounds are deep and circulation around the wound is compromised, this product might also be of use for other types of wounds[124].

SureDerm® is obtained by sequential treatments with dispase followed by Triton X-100[125,126]. The enzymatic treatment with dispase removes the epidermal layer. It is freeze-dried and stored at temperatures of 2°C to 8°C. SureDerm® can be applied

together with an STG (one-step), but there is a high risk of infection. Histologic examination showed that this product is completely absorbed within 4 months[125,126].

Product	Major Substances	Origin	Contraindications	Price (US $/cm²*)
Alloderm (Life Cell Corp.)	Collagen (+elastin) matrix	Human donor skin		10
DermaMatrix (Synthes)	Collagen (+elastin) matrix	Human donor skin		10
GlyaDerm (Euroskinbank†)	Collagen + elastin matrix	Human donor skin		2
Integra (Integra LifeSciences Corp)	Collagen + glycosaminoglycan matrix + silicon layer	Bovine achilles tendon + shark cartillage	Known allergy to bovine collagen or silicone	8
Matriderm (Skin&Healthcare)	Collagen matrix covered with elastin fibers	Bovine dermis and ligamentum nuchae	Known allergy to bovine collagen or elastin	6
Pelnac (Kowa Company)	Collagen matrix + silicon layer	Bovine dermis	Extremities of children and patients susceptible to keloid formation. Known allergy to silicone	4
Permacol (Tissue Science Laboratories Plc.)	Collagen (+elastin) matrix	Porcine dermis	Known allergy to porcine collagen	14
Renoskin (Groupe Perouse Plastie)	Collagen matrix + silicone outer layer	Bovine	Known allergy to bovine collagen or silicone	8
SureDerm (Hans Biomed Corp.)	Collagen (+elastin) matrix	Human donor skin		3
Terudermis (Terumo Corp.)	Collagen matrix (+silicone layer)	Bovine dermis	Known allergy to bovine collagen (and silicone)	5

* Price estimates were obtained by e-mail contact with the companies (December 2008) or publically available on the internet. Prices depend on the country and the size of the product. Not all products are available worldwide. NA, price not available.
† Nonprofit organization.

Table 1. Dermal matrices commercialized for acute full thickness wounds and reconstructive surgery.

Hyaluronic acid

A third group of dermal matrices consist of hyaluronic acid (**Table 2**), which is normally produced by fibroblasts[73,127,128]. After purification, hyaluronic acid is identical in all species and phyla[129], and it seems to have a major impact on scar-free fetal wound healing[11]. It can be obtained from Streptococcus fermentation or extracted from rooster combs. It is used in wound healing, ophthalmology, and joint surgery. Some of the frequently used dermal fillers are also based on hyaluronic acid[63]. Hyaluronic acid can be esterified to obtain a stable cross-linked matrix which will not liquefy and will postpone degradation[62], permitting the application as dermal matrix. Hyaluronic acid is available as a scaffold for keratinocytes (cf. supra: Laserskin), an acellular dermal matrix (Hyalomatrix), and as a cellular dermal matrix (cf. infra Hyalograft-3D). Several variations with different degradation profiles (up to 4-5 weeks) are currently being investigated, even in combination with endothelial cells[130,131]. Some of the degradation products modulate wound healing[62] and are proangiogenetic[129,132].

Currently available cellular dermal substitutes - dermal equivalents

The cellular dermal substitutes or “dermal equivalents,” (**Table 2**) are obtained by culturing fibroblasts on a collagen, hyaluronic acid, or synthetic scaffold. These fibroblasts will synthesize extracellular matrix components and growth factors. The currently available cellular dermal matrices mostly contain allogenic cells, improving healing by production of cytokines, etc. The same principles can also be used for

culturing autologous cells, but the long cultivating time limits the use to chronic wounds and severe burns. Although allogenic fibroblasts themselves do not induce immunogenic reactions in the host, they may accelerate second-set rejection[133].

Allogenic fibroblasts can be obtained from neonatal foreskin, human donor skin, or surgical "leftovers" (after abdominoplasty, breast reductions, etc.)[35,134 -137], but infection transmission should always be considered. Autologous cells can be obtained by a skin biopsy, but also of a liposuction aspirate, reducing donor-area morbidity[35], or eschar obtained through debridement of burn wounds[35]. Nevertheless, those "alternative" fibroblasts showed more contraction *in vitro*[35,138].

The biological temporary dressing Dermagraft-TC ("Transitional Covering")[137], now named TransCyte, is a porcine collagen-coated nylon mesh with non-viable-cultured foreskin-derived dermal fibroblasts covered with silicone[137,139,140]. A modification of Dermagraft-TC/Transcyte got the confusing name Dermagraft, which contains viable allogenic neonatal foreskin fibroblasts on a bioabsorbable polyglactin mesh that disappears after 3 to 4 weeks[135,141,142]. New elastin was not detected after 1 year. Dermagraft showed to be effective in the treatment of chronic wounds like diabetic foot ulcers[135,143]. The biggest disadvantage is that multiple applications might be necessary, and therefore, the classification as dermal substitute remains questionable.

Hyalograft 3D[127,130] is based on esterified hyaluronic acid. Fibroblasts are cultured on this non-woven mesh creating a 3D cellular matrix *in vitro*[131,144]. Two-step interventions, where dermal hyaluronic acid matrices were covered with Laserskin (autologous keratinocyte cultures), already proved useful for chronic and acute full thickness skin defects[131,145,146]. Fibroblasts were also cocultured with human umbilical vein endothelial cells, leading to the *in vitro* development of capillary-like structures, improving integration[130].

Product	Major Substances	Origin	Contraindications	Price* (US $/cm^2*)
Apligraf (Organogenesis)	Collagen + glycosaminoglycans + allogeneic fibroblasts + allogeneic keratinocytes	Bovine tendon + neonatal foreskin (cells)	Infected wounds, known allergy to bovine collagen, hypersensitivity to agarose shipping material	32
Dermagraft (Advanced BioHealing)	Polyglactin mesh + fibroblasts	Synthetic mesh + neonatal foreskin (cells)	Infected wounds	38
E-Z Derm (Brennen Medical)	Collagen matrix	Porcine dermis	Known allergy to porcine collagen	3
GraftJacket (Wright Medical technology Inc.)	Collagen (+elastin) matrix	Human donor skin		NA
Hyalograft (Fidia Advanced Biopolymers)	Hyaluronic acid + allogeneic fibroblasts	*Streptococcus* fermentation + neonatal foreskin (cells)	Hypersensitivity	NA
Hyalomatrix (Fidia Advanced Biopolymers)	Hyaluronic acid + elastomeric outer layer	*Streptococcus* fermentation	Hypersensitivity	3
Oasis (Healthpoint)	Collagen matirx	Porcine small bowel submucosa	Known allergy to porcine collagen	4
OrCel (Forticell)	Collagen + allogeneic fibroblasts + allogeneic keratinocytes	Bovine tendon + neonatal foreskin (cells)	Known allergy to bovine collagen	Currently not available
Primatrix (Tei Biosciences Inc.)	Collagen matrix	Foetal bovine dermis	Known allergy to bovine collagen	NA

* Price estimates were obtained by email contact with the companies (December 2008) or publically available on the internet. Prices depend on the country and the size of the product. Not all products are available worldwide. NA, price not available.

Table 2. Dermal or combined skin replacements commercialized for the treatment of chronic wounds and/or partial thickness acute wounds (Burns, donor sites, etc.), epidermolysis bullosa.

Combined skin substitutes

All combined skin substitutes or composite grafts are manufactured by culturing keratinocytes on a dermal layer, often containing living fibroblasts (**Figure 1, Table 2**). Several techniques can be used to obtain dermal matrices, but clinical studies remain rare (especially long-term follow-up)[147]. The first commercialized combined skin substitutes, based on collagen scaffold, already date from the late 80s, and are based on the models of Bell and Boyce.

The model of Bell

This combined skin substitute was obtained by incorporating living fibroblasts in a collagen solution with serum, resulting in a resistant and impenetrable layer[115,136,148-155]. The keratinocytes are cultured on top, forming an epidermal layer, without forming a real dermoepidermic junction. This method is applied in severe burns with a "take" ranging from zero to maximum 70%[148,151]. *In vivo*, an STG or keratinocyte culture needs to be grafted on top during a second operation, resulting in a better take-rate and a better esthetical result[150]. This technique with living neonatal foreskin-derived keratinocytes and fibroblasts is commercialized as Apligraf (Graftskin)[115,136,152-154]. It has demonstrated the ability to produce a number of cytokines and growth factors, and it acts very much like human skin[155]. A more advanced product can be obtained by adding melanocytes, and a hypodermis composed of preadipocytes and adult adipocytes. It might even be combined with hair follicles. The biggest disadvantages are the limited viability, the high cost, and the need for extensive viral screening[156].

The model of Boyce

Another model is cultured on a matrix of bovine collagen and glutaraldehyde[157-162]. The fibroblasts and keratinocytes are each cultured on one side of the sponge, forming a complete dermoepidermic junction *in vivo*[157-159]. To assure nutrition of the epidermal cells, before vascularization through the dermis, the epidermal cells are exposed to the nutrients in the culture medium. This dermal equivalent with autologous material or "Cultured Skin Substitute" is used clinically since 1989. After the skin biopsy, the preparation takes 20 to 30 days[161-164]. Clinical results were cosmetically satisfactory and similar to STGs. This model is also available as OrCel with living neonatal foreskin cells[165,166]. OrCel serves as an absorbable biocompatible matrix that provides a favourable environment for host cell migration, containing several cytokines and growth factors. Resorption appears to take place gradually, with no remnants 2 weeks after treatment. There are limited clinical data available for this product, but large clinical trials are ongoing[165].

Currently investigated combined skin substitutes

Several dermal matrices, like acellular dermis, collagen matrices, human solidified plasma, and matrices produced by human umbilical vein endothelial cells were already used as

template for these composite grafts *in vitro* and/or *in vivo*[94,95,115,130,136,148 -162,167-179]. Several of these composite grafts have been used to study the skin physiology. In particular, the dermoepidermic junction is necessary for the survival of the epidermal layer[36,170]. The presence of fibroblasts increased the epidermal differentiation and resulted in increased graft take, less contraction, and enhanced vascularization[171]. Skin also contains melanocytes, hair follicles, and sweat glands, which are very difficult to replace. Some research groups are testing composite grafts with melanocytes and even Langerhans cells clinically, but the results remain to be optimized[180,181].

Discussion and conclusion

Because of the advancements in tissue engineering, the treatment possibilities for skin defects evolved the last three decades from mainly preventing infection to the use of biologically active products and skin substitutes. These skin replacements can be distinguished from (biological) dressings because they become incorporated in the healing wound and consequently, do not need removal. However, despite of being commercialized as permanent skin replacements, most of these products are completely replaced by autologous tissue within a couple of weeks or months due to normal biological "renewal" processes. Even the most popular skin replacement Integra, is not detectable anymore 5 weeks after application[54]. Yet, those products are often referred to as "permanent," making it difficult to differentiate them with other "technically" identical products, currently commercialized as "temporary" skin substitutes or biological dressings. Nevertheless, these skin replacements will have a certain influence on the healing process and the quality of the remaining scars. They serve as a matrix for cellular invasion from the surrounding tissue, and some of them will also stimulate healing, similar to biological dressings. To create a more transparent classification of all those "permanent" surgically applied skin replacements, we propose the following categories: epidermal substitutes, dermal substitutes, and combined skin replacements. The dermal substitutes are sub-divided in several categories, depending not only on the main substance (and its origin) but also on the presence of living cells. A fourth group will consist of skin and soft tissue composite grafts (but these products are still in the experimental phase).

The advantage of cultured keratinocytes as epidermal replacement is that closure of the burn wounds is possible with autologous cells even in severe burns where the donor sites for STGs are limited. The main disadvantage is the absence of a dermal layer, which leads to blistering, hypertrophic scarring, and severe contractions when applied to deep wounds. The long cultivation time and the high costs also limit the

clinical use. Keratinocytes can also be used to stimulate healing of the donor sites of STGs, which facilitates early “recropping”.

The dermal substitutes have the main advantage of replacing the dermis, which will lead to a more aesthetical and functional outcome. Nevertheless, the epidermal layer also needs replacement, by thin STGs or keratinocyte cultures. This layer can be restored during the same operation, or after ingrowth of the dermal matrix, which usually takes 1 week to 3 weeks. This two-step procedure is necessary for several matrices, because of the slow vascularization. Highly porous scaffolds with a very diffuse matrix may be more rapidly penetrated by budding neocapillaries than more densely formed scaffolds. However, the turnover of the porous scaffolds may be so high their role as dermal substitutes is questionable. Further research is needed to differentiate between the currently available dermal matrices, and strategies need to be developed to accelerate the invasion of the matrix by fibroblasts and vascular structures from the surrounding tissue. The main goal is to obtain a more optimal healing process (less infections, better “take”) and a further reduction of the scarring and contour deformities.

The combined skin substitutes are a combination of the two previous groups and should be able to restore a full thickness defect in a one-step procedure. Nowadays, those products are mostly used in chronic wounds (with allogenic cells) but often need repeated applications. Because of the living cells and often complicated manufacturing processes, those products remain very expensive (up to 35 US $ per square centimeter).

In full thickness skin and soft tissue defects, restoration of the dermis and epidermis will often be insufficient, because of the remaining depression compared with the surrounding tissue. Currently, two options are available: reconstruction by flap surgery (primary or secondary) or secondary soft tissue augmentation underneath the primary healed skin (dermal fillers, autologous fat transplantation, or prosthesis). Especially, the autologous fat transplantation (“lipofilling”) is gaining importance because subcutaneous fat is present in sufficient amounts in the majority of people, and it is easily accessible[182]. In the future it might be possible to combine adipocytes with the skin substitutes to close the deep defects in one operation[7]. But accelerated vascularization becomes even more important[163,183,184]. Promising allogenic cells are human umbilical vein endothelial cells[185,186] and human dermal microvascular endothelial cells[183,184]. Stem cells (obtained from the bone marrow, subcutaneous fat, etc.) may gain interest to create this vascularized skin-fat-matrix, because of their ability to divide and renew themselves over long periods of time, to differentiate into various cell types, and their relatively easy isolation and expansion[187-194]. The use of stem cells for acute wounds (burns, etc.) will probably remain limited because of the time needed for cultivation. Ready-to use, off-the-shelve products will probably remain more useful for certain indications.

Other futuristic developments include genetic modifications of transplanted cells to improve wound healing transiently and to deliver gene products systemically[160,163,184,195-197]. Genetic modification of cells within skin substitutes can hypothetically be used to overcome limitations in anatomy and physiology, resulting

in skin substitutes with greater homology to native human skin and improved performance (improving vascularization, etc.).

In conclusion, 200 years after its discovery, the STG technique remains the preferred method for burn coverage for most surgeons. The currently available skin substitutes and biological dressings are very expensive and their clinical efficacy remains a topic of controversy and continued research. To our knowledge, there are no large controlled, randomized studies attesting the clinical efficacy of any of the currently available dermal substitutes. However, evidence is increasing that wound bed preparation and the use of dermal substitutes contribute to a more optimal wound healing with improved quality of scars, reduced rate of contraction and ultimately, a better quality of life. Researchers continue their quest for the ideal skin substitute, and in the future it should be possible to create such an advanced skin substitute, containing melanocytes, hair follicles, and sebaceous glands. The available products remain rather expensive, because of commercial incentives, high manufacturing, shipment, and storage costs. Nevertheless, accelerated healing and closure of the wound will reduce the labour-intensive dressing changes, hospital stay, and the need for reconstructive surgery. Until the optimal off-the-shelve skin substitute becomes available, the burn surgeon can improve aesthetic and functional outcome by choosing from the gamut of currently available scaffolds for bilayered skin restoration. This classification has intended to facilitate clinical and cost-economic decision making.

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CHAPTER 6

BURN SCAR ASSESSMENT - A SYSTEMATIC REVIEW OF DIFFERENT SCAR SCALES

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N. BRUSSELAERS
A. PIRAYESH
H. HOEKSEMA
J. VERBELEN
S. BLOT
S. MONSTREY

Background

Scars can be devastating and disfiguring, because they are clearly visible, stigmatizing, and permanent reminders of the initial accident or surgical event. Yet, there is still no consensus about the optimal scale or tool to assess the characteristics and evolution. Our aim was to evaluate the clinical importance of scar scales specifically developed for burn scars.

Materials and methods

The systematic literature search involved PubMed and the Web of Science (including Science Citation Index).

Results

The search resulted in 29 articles (including seven reviews) dealing with a new, modified, or validated scale. Scar scales assess several characteristics, of which color, pliability, and thickness were considered the most important. Physical limitation, pain, and pruritus are often more disturbing than the appearance of the scar, and are therefore also introduced in scar evaluation, as well as the interference with daily life activities (e.g., psychologic impact).

Conclusion

In contrast to the more objective scar assessment tools, scar scales usually cover more aspects of the scars and are less time-consuming in clinical practice. However, no strong conclusions can be made about their efficacy and validity. In addition to digital photography, scar scales are a valuable instrument in the clinical evaluation and follow-up of scars.

Introduction

Due to the improvements in burn treatment as provided in highly specialized burn centers, more patients with deep and extended burn injuries do survive nowadays[1-3], resulting in a larger group of patients with more extensive scar formation[4]. Scar formation depends on several variables, including the wound treatment, the depth of the burn, the skin type and age of the patient, the healing process (inflammation, infection, etc.) but also on the application of preventive measures[5, 6]. As a rule, wounds that are not healed within 2-3 weeks are considered most at risk for excessive scar formation[7].

Because of the relatively high prevalence of unfavorable scar formation after burns, most studies on scar assessment and scar treatment are focused on the burn scar[7-10]. Surgical and dermatologic scars will rarely result in extensive scar formation, and since the impact of scar complications strongly correlates with the dimension of the scar (e.g., pain, itching, and fragility), the impact of these types of scars is usually more limited, although also less well studied[11]. Therefore, burn scars are probably the scars with the highest impact on the quality of life[12-20]. Both physical and psychologic effects related to excessive scarring may hamper the quality of life, including the often lengthy, painful treatment, often resulting in still a suboptimal result[11, 12, 19, 21-25]. Scars may cause pain, itching, and discomfort; and contractures may also constrict mobility. The integration of patients with hypertrophic scars in a society where well-being, individuality, and external appearance have become increasingly important might also be troublesome[12]. It has been demonstrated by many authors that burn scars, because of their clearly visible and stigmatizing appearance, may have a major psychologic impact, comparable to other chronic (skin) diseases[11, 12, 22, 26-28]. A study of Balci et al. analyzed the quality of life in patients with hypertrophic scarring and keloids, and found a similar impairment as in patients with psoriasis[22]. Brown et al. identified five main areas of impact in patients with excessive scarring resulting in coping behavior to hide or compensate the scars: the physical comfort and functioning, confidence in the nature and management of the condition, acceptability to self and others, social functioning, and emotional well-being[11]. They concluded that scarring has a major influence on a patient's psychologic morbidity and behavior, and has important implications for clinical practice. Van Loey et al. described how scars may contribute to social anxiety and posttraumatic stress syndromes, since pressure garments or red and disfiguring scars can attract a lot of attention from other people, which may induce feelings of shame[12, 29]. Several preventive measures and treatments have been proposed to decrease pathologic scar formation, and multiple invasive and noninvasive treatment modalities have been introduced[5, 30-34]. Although scar assessment seems essential, this is still a neglected area, and there is still no consensus on the ideal method of scar evaluation, in spite of the many scales and tools that have been developed during the last decades[34]. Adequate assessment of scars is, however, important in the clinical evaluation and follow-up, but it is also essential to compare different wound or scar treatment modalities[35-41]. Moreover, for

medico-legal reasons, an objective scar evaluation can be required, e.g., for reimbursement of treatment and proof of disability.

Scar evaluation can be performed by rather simple, paper-and-pencil scar scales assessing several variables, usually by purely subjective word-descriptions (red, elevated, etc.), but also by using technically advanced and objective devices (scar tools) analyzing one or more variables in a more reproducible way (spectrometry, ultrasound etc.)[36, 39, 40, 42-50]. The objective of this study was to provide an analysis and critical overview as to which scar scales have been developed to assess the physical aspect of burn scars, and what their role is in burn assessment.

Methods

Criteria for considering articles for inclusion

Initially, only articles dealing with burn scar assessment scales as a major topic were included. In order to obtain an overview of all scar scales that can be used for the evaluation of a burn scar, several articles about scar assessment in general (e.g., for surgical wounds) were also implemented. Articles comparing the influence of different treatments on scars were excluded because they were irrelevant to this study. Quality of life scales and other scoring systems assessing the psychologic and ergonomic impact of the scar (or other pathologies) were not included in this review because they are aspecific and generally not used by the treating physician assessing the scar or the resulting physical impairment. These quality of life scales are nevertheless important in the multi-disciplinary approach of the burn patient, as an addition to the physical assessment of the scar. Studies about advanced scar tools such as ultrasound and colorimetry are not the subject of this study, and are discussed elsewhere[34].

Search methods

We conducted a systematic literature search involving PubMed and the Web of Science (which also contains major Congress abstracts)[51, 52]. The Cochrane Library did not include relevant articles. We searched PubMed from 1960 to 2009 (date of search 18 February 2009), using the Medical Subject Headings (MeSH) ‘Burns’ and ‘Cicatrix’ (**Figure 1**). This search retrieved 1974 articles, and was narrowed down to articles with those terms as ‘major topic’ and specific types of burns were excluded (the MeSH ‘burns’ was not exploded) (n 1⁄4 928 articles). Limits were set to English, French, and Dutch articles about human studies, resulting in 597 studies. The MeSH terms ‘surgical flaps’ and ‘neoplasms’ were used to exclude all irrelevant articles dealing with flap surgery (n 1⁄4 78) and cancer development in burn scars (n 1⁄4 91). From the remaining 428 articles, 67 articles were manually selected based on title and abstract, resulting in 15 articles corresponding with the inclusion criteria (**Figure 1**)[36, 37, 39, 42, 53-63]. We also searched the Web of Science on the terms ‘scar’ or ‘cicatrix’ and ‘burns’ or ‘burn’, but even after language selection, this led to almost 44,000 hits. Therefore, some stricter combinations were performed (on ‘burn’, ‘burns’, ‘thermal

injury', 'cicatrix,' and 'scar'), resulting in 443 articles, and after hand searching this led to three additional articles[35, 64, 65].

We finally submitted the relevant articles to a Science Citation Index search, and the reference lists were searched, resulting in 11 more articles[38, 40, 66-74]. In total, 29 articles are included dealing with scar assessment, of which 22 are original articles (reporting on new, modified, or validated scar scales) and seven are review articles or editorials[35-40, 42, 53-74].

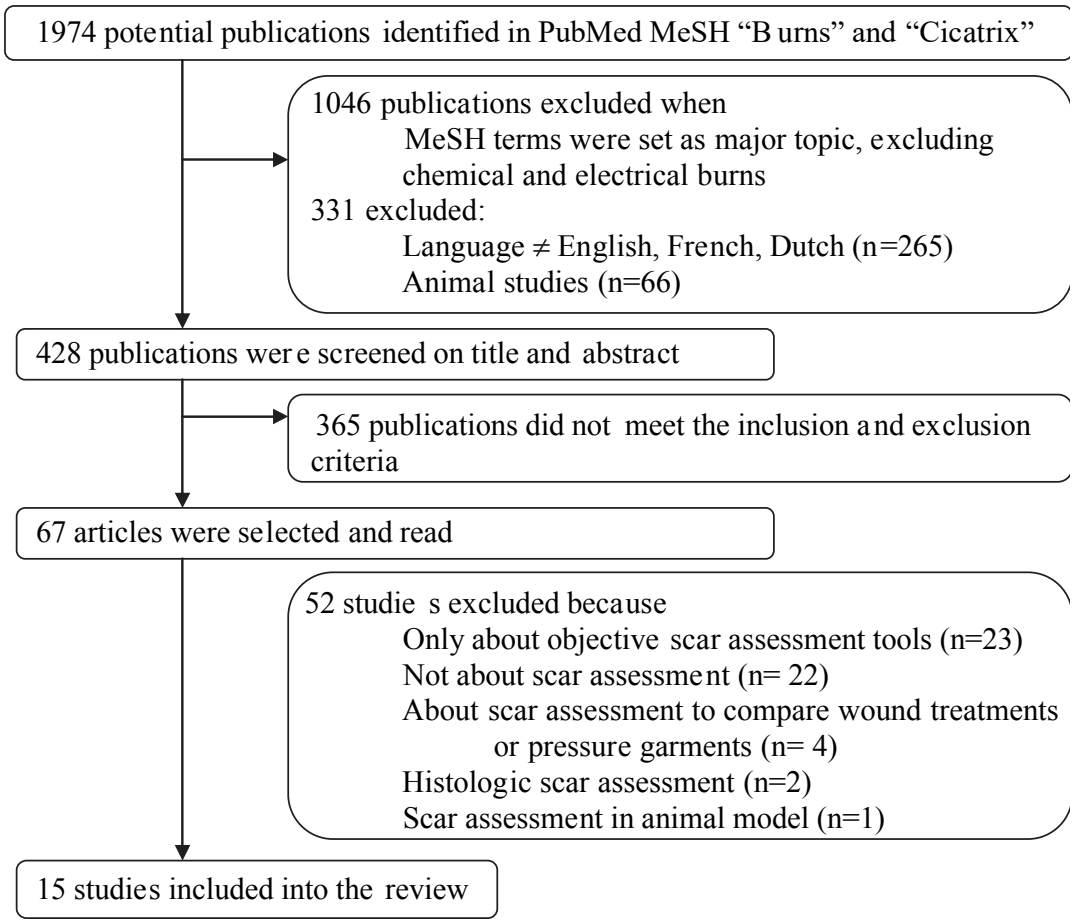

Figure 1. Flowchart.

Quality assessment

When available, statistical data were collected for comparison of validity and reproducibility of the different scoring systems. Two measures for reliability and reproducibility were used in this paper, which were most commonly reported. The inter-rater variability (or interclass correlation) expresses the differences in measuring between several assessors. The single-rater or intra-rater variability assesses the variation when one single assessor rates the same scar multiple times, but the mathematical principle is the same as for the inter-rater variability. An inter-rater variability of 0.9 corresponds with a 90% identical assessment by all evaluators and may be considered good. An inter-rater variability between 0.7 and 0.9 means there already is a large variation between the different assessors (the scar is not rated equally severe). Low scores (<0.7) correspond with insufficient correlation between the different assessments, reflecting a low reproducibility.

Results

Thirty years ago, the first scar scale was introduced by Garcia-Velasco et al., who assessed color, consistency, and thickness of the hypertrophic burn scar in children (**Table 1**)[69]. These characteristics all received a score from 1 to 3, but these were not combined into a single score. This scale was not appropriate for assessment of other types of scars, e.g., after surgery. In 1988, Smith et al. scored irregularity, thickness, and color, and they proposed color photographs for burn scar assessment, and also assessed the overall cosmetic disfigurement (**Table 1**)[70]. This scale cannot be used reliably by fewer than three assessors (for four person-assessment the inter-rater variability was up to 0.94)[35, 36, 70]. Despite the poor single-rater reliability for the different subscores (which varied between 0.39 and 0.79), Smith et al. were pioneers in measuring cosmetic disfigurement in a reliable way, leading to more suitable metric scales in the following years. In 1989, Leung et al. developed a similar classification system for scars but the consistency of the scale was not reported[63].

Author (year)	Variables	What's new?
Garcia-Velasco (1978) [69]	(i) Color: white- pink or purple - red (grade 1-3); (ii) consistency: soft- moderately hard-hard (grade 1-3); (iii) thickness: fla- slightly raised-prominent (grade 1-3)	
Smith (1988) [70, 73]	(i) Surface: smooth- partly irregular-mostly irregular; (ii) thickness: slight- moderate-severe; (iii) color: no difference-slightly darker-much darker, (iv) cosmetic disfigurement clothed/not clothed: none-very slight-slight-moderate-severe-very severe	- Standardized photographs to aid comparison
Brou (1988) 'Inventory of Potential Reconstructive Needs' (IPRN) [68]	Systematic thorough documentation of functional and cosmetic burn sequelae in children (contractures etc.)	- Template for systematic planning of reconstructive procedure
Leung (1989) [63]	(i) Color: slightly pink → deep purple (grade 1 → 5), (ii) consistency: very soft → very hard (grade 1/ 5), (iii) thickness: very thin → very thick (grade 1 / 5)	- classification used for the evaluation of an objective scar assessment tool (laser Doppler flometer)
Sullivan (1990) [53, 56, 60-62] 'Vancouver Scar Scale' or VSS *	(i) Pigmentation: normal-hypo-hyper (0-2 points); (ii) vascularization: normal-pink-red-purple (0- 3 points), (iii) pliability: normal-supple-yielding-fir- banding-contracture (0-5 points); (iv) height/thickness: normal; < 2; < 5; > 5 mm (0- 3 points) → total sum between 0 and 13	- Defind body diagram was used
Baryza (1995) [61] 'Modifie VSS *'	(i) Pigmentation: normal-hypo-mixed-hyper; (ii) vascularization: idem VSS * [53] ; (iii) pliability: idem VSS* [53]; (iv) height/thickness: idem VSS* [53] → total score between 0 and 14	- Extra pigmentation category: mixed pigmentation
Yeong (1997) [54, 55] 'Seattle Scar Scale'	(i) Surface irregularity: from smooth to extremely rough (- 1 to 4); (ii) thickness: from thinner to extremely thick (- 1 to 4); (iii) border height: from depressed to extremely raised (- 1 to 4); (iv) color: hypopigmented to hyperpigmented (- 1 to 4) → total sum between - 4 and 16 points	- 24 standardized photographs to aid comparison - Negative scores for hypopigmentation, depressed scars etc.
Crowe (1998) [70, 73] 'Hamilton Scar Scale'	(i) Surface irregularity (bumpy or irregular): smooth - 1/4 irregular - 1/2 irregular - 3/4 irregular - majority of the scar irregular (0-4 points) (ii) thickness: none-slight-moderate-severe (0- 3 points); (iii) color: normally pigmented/mature - light to medium pink - deep pink to light red - medium to deep red - purplish (0- 4 points); (iv) vascularity: normal or paler - slightly darker - darker - much darker (0- 3 points) → total sum between 0 and 14 points	- Analyses of photographs of the scar
Beausang (1998) [66, 72] 'Manchester Scar Scale'	(i) Color: perfect-slight mismatch - obvious mismatch - gross mismatch (1- 4 points); (ii) matte/shiny (1- 2 points); (iii) contour: flush with surrounding skin-slightly proud/ indented-hypertrophic-keloid (1- 4 points); (iv) distortion: none - mild - moderate - severe (1-4 points); (v) texture: normal - just palpable - fir- hard (1- 4 points); (vi) overall assessment: VAS (0-10 points)** → total score between 5 and 28 points	- VAS** to assess the overall appearance of the scar

(Continued)

(Continued)

Author (year)	Variables	What's new?
Nedelec (2000) [56] 'Modifie VSS'	(i) Pigmentation: normal- slightly ...- moderate...- severely increased or decreased (0- 3 points); (ii) vascularity: idem VSS (0- 3 points); (iii) pliability: normal-supple-yielding-firadherent (0- 4 points); (iv) height: normal, 1-2, 3-4, 5-6, > 6 mm (0- 4 points); (v) itching and pain: by VAS*; (vi) pain medication → total sum between 0 and 14 points + 2 VAS *	- Includidg itching and pain - A total score was not made
Fisher (2001) Modifie IPRN (MIPRN) [57]	Systematic thorough documentation of functional and cosmetic burn sequelae in children (contractures etc.) including pigmentation change, hypertrophic scarring, scar pockets. Also: eyebrow alopecia, heterotopic ossificion, nail deformities, inter-phalangeal fleion and extension contractures, microstomia, burn syndactyli etc.	- Some specific scar sequelae were added to the IPRN
Draaijers (2004) [59, 67] 'Patient and Observer Scar Assessment' *** (POSAS)	(i) Color (P) (0-10 points); (ii) pigmentation (O) (0-10 points, and hypo-mixed-hyper); (iii) vascularization (O) (0 → 10); (iv) pliability (P+O) (0-10 points); (v) thickness (P+O) (0-10 points); (vi) relief (P+O) (0-10 points) (vii) itching (P) (0-10 points); (viii) pain (P) (0-10 points); → total sum between 0 and 80 points	- Separate scoring by patient (P) and physician/ observer (O) - Van de Kar added 'surface area' (O): expansion-contraction (0→10), and an overall opinion (P) (0→10**) [67]
Smith (2004) (Long distance scar assessment by videoconference)	(i) Color: pale-pink-red; (ii) thickness: yes-no (if yes, where?); (iii) contractures: yes-no (if yes, where?); (iv) range of motion: restricted or not; (v) general level of activity: restricted or not; (vi) breakdowns of graft sites: yes-no (if yes, where?)	- Long-distance assessment by videoconference - Includes the activity level and physical impairment (range of motion)
Masters (2005) [54, 55] MAPS (Mapping assessment of scars and photographs	(i) Surface irregularity: cf. Yeong [54] ; (ii) thickness: cf. Yeong [54] ; (iii) border height: cf. Yeong [54]; (iv) color: white-normal-pink-red-purple-dark purple (- 1 to 4); (v) pigmentation: hypo - normal - hyper (- 1 to 1); (vi) pain and itching	- Photographs are taken of each scar - Body chart with grid for marking scar site (for relocalization of the scar site). - Colour is not subdivided anymore in vascularity and pigmentation - Applicable for all scars
Rea (2006) [58] Reconstructive needs assed by patient and surgeon ***	(i) Localization of scarring (body chart) (P+O); (ii) do you consider reconstructive surgery for any area? (P); (iii) ranking of areas considered for surgery (P+O); (iv) preferred reconstructive procedure (O)	Priorit izing reconstructive needs
Forbes-Ddchart (2007) [60] 'Modifie VSS' *	Idem VSS * [53]	Two series of standardized photographs (Caucasian and Aboriginal) were introduced to aid color assessment [60]
Singer (2007) 'Stoner Brooks Scar Evaluation	(i) Width: > 2 mm- ≤2 mm (0- 1 points); (ii) height: elevated - depressed (0- 1 points); (iii) color: darker - same or lighter (0- 1 points); (iv) hatch or suture marks: present - absent (0- 1 points); (v) overall appearance: poor - good (0- 1 points) → total score from 0 to 5 points	- Applicable for all scars - Assessing hatch and suture marks - An increasing score correlates with an improving scar

Total scores are noted within (...) when it is optional to calculate them.
*VSS ¼ Vancouver scar scale, also named Vancouver burn scar assessment scale or Burn scar index.
**VAS ¼ visual analogue scale.
***Assessment by patient (P) or observer/physician/surgeon (O).

Table 1. Historical milestones in the development of scar scales

The Vancouver scar scale and its modifications

The first validated and still widely used scar assessment scale is the Vancouver Burn Scar Assessment Scale or Vancouver Scar Scale (VSS) developed by Sullivan et al.[35, 36, 38-40, 42, 53, 56, 66, 71]. They scored pigmentation, vascularity, pliability, and scar height/ thickness, leading to a total score between 0 and 13 points (**Table 1**). The VSS proved to be insufficient for large and irregular scars where hypertrophy, pliability, and color are not homogenous[38, 72]. Therefore, this scale is strongly investigator-dependent, using pure word descriptions, and it does not locate the test site within the scar, which is necessary for follow-up[59, 67]. The numeric scoring of each variable is also questioned because not all variables are considered to be equally important for

each patient or physician. Another problem already identified by Sullivan et al. was the lack of registering itching and pain. Several modification were therefore described (**Table 1**)[56, 59, 61]. Baryza developed a pocket size VSS to aid in scoring the scar and to increase the staff compliance. The inter-rater variability was 0.81[61]. Nedelec et al. adjusted the VSS to increase the reliability and validity although training in the use of this scale was required. They tried to improve the quality of the subscales (without making a total score). Nedelec et al. were also the first to implement the opinion of the patient by assessing pain and itching[56] However, inter-rater variability was poor for the separate variables: 0.20 to 0.42 (when combined: 0.53). Extending the use of this scale to other types of scars remained difficult. Forbes-Duchart et al. modified the VSS because it was not culturally sensitive for their Aboriginal patients[60]. They developed two color scales based on photographs to assess the vascularization, one for Caucasian and one for Aboriginal skin. Nevertheless, the vascularity and pigmentation subsets had poor reliability for the Aboriginal patients (with even negative correlating scores for pigmentation). The inter-rater variability for the total score applied to a mixed population varied from 0.76 to 0.84[60]. The authors therefore suggested to use 'light', 'medium', and 'dark' skin, for patients of differing skin tones, regardless of race[60].

The Seattle scar scale and MAPS

To obtain a more uniform scoring, Yeong et al. (1997) proposed a numeric scale based on a set of 24 standardized color pictures, assessing scar surface, thickness, border height and color differences between the scar and the adjacent normal skin[54]. The scale ranges in whole numbers from -1 to 4, by increasing severity with zero indicating normal (**Table 1**), and had an inter-rater variability between 0.85 and 0.97[54]. Although the authors themselves did not recommend to add the subscores, the biggest criticism was that negative values for parameters, which are in the opposite range of the hypertrophic score (hypo-pigmentation, atrophy), yielding an 'improved' total score[54].Masters et al. optimized the assessment of Yeong et al. in 2005 by adding a localization technique [matching assessment of scars and photographs (MAPS)] to describe the general appearance of a scar rather than a specific spot[37, 55]. The photographs enabled long-term follow-up over a period of months[55]. The interrater variability was poor (between 0.55 and 0.78) for border height, thickness, and color, and between 0.25 and 0.40 for surface[55].

The Hamilton scar scale

Crowe et al. introduced the Hamilton scale, which is a modified version of the scale of Smith et al.[35, 42, 70, 73]. They did not apply the controversial negative scores used in the Seattle Scar Scale, but did use scar irregularity, which is not scored in the VSS (**Table 1**). This scale was reliable, even when used by novice therapists, with an inter-rater variability between 0.73 and 0.89[73].

The Manchester Scar Scale and other Visual Analogue Scales

In 1998, Beausang et al. developed a quantitative rating scale including scar contour, radiance (matte or shiny), color, texture and distortion[35, 38, 58, 66]. Photographs and a visual analogue scale (VAS) were used for scoring the general appearance (**Table 1**). It avoided the difficult color determination (pigmentation or vascularization), leading to an inter-rater variability of 0.87[35, 36, 38-40, 66].

Bayat et al. were the first to include 'size of the scar' and 'multiple scars' in their scale[38, 72]. These data are part of the 'Manchester Proforma', which is a standardized form for clinical and outpatient follow-up of burn patients. A standardized color photograph was taken at each consultation as a reference to evaluate effectiveness of treatment. It was especially useful for small burn scars to detect the evolution of hypertrophic or keloid scars, but it does not assess vascularity[72].

Martin et al. only used two VAS scales to assess the opinion of the patient about his/her scar, and how he/ she thinks the scar is perceived by others[62]. A computerized visual analogue scale was also used in the study of Duncan et al. They compared the results of the VAS with a ranking system[65]. This scar scoring and ranking system appeared to be consistent, reliable, valid, and feasible for both research and clinical settings, with inter-rater variability for small linear scars (created in healthy volunteers) of 0.90-0.92 for the VAS, and 0.80-0.86 for the ranking[65].

The Patient and Observer Scar Assessment Scale (POSAS)

Draaijers et al. developed a double numeric scoring system, one for the physician and a separate score for the patient (which cannot be used for young children): the Patient and Observer Scar Assessment Scale[38, 59, 67, 71]. The patient scale contained six parameters, while the observer scale only scored five parameters (**Table 1**). Van de Kar et al. added change in scar surface area (expansion, contraction, mix) to the observer scale for a more complete evaluation of linear scars[67]. There was a good correlation between both scales, which are very useful in clinical practice. Especially itching was shown to be significant for the patient. The internal consistency of the observer scale was 0.86[67].

Long distance assessment

Videoconferencing has become a routine technique for the post-acute burn care of children in Queensland (Australia) because of long travel distances to the hospital[64]. Therefore a telemedicine scar-assessment form was developed by Smith et al. (2004) to measure the following six variables (**Table 1**): color, scar thickness, contractures, restricted range of motion (patients followed verbal instructions), restricted general level of activity (also described by the patient or parents), scar breakdown (assessment of graft sites with close-up live images þ verbal information). Agreement between consultants was moderately high, and comparable to face to face assessment (0.84 and 0.85 respectively)[64].

The Stony Brook scar evaluation scale

Singer et al. developed several scales, to assess wound healing, and to facilitate histologic evaluation of scar biopsies[74-77]. Eventually, Singer et al. also developed a scar scale, because their wound evaluation score (intended to measure the short term outcome of wounds) had been used regularly on scars[74, 78] despite of useless variables e.g. the presence of wound margin separation. Therefore, they also developed this scar scale, scoring five parameters: width, height, color, hatch or suture marks and overall appearance (**Table 1**). The inter-rater variability was between 0.75 and 0.92[74].

Reconstructive needs

Another type of scar scales was developed specifically to assess the need for reconstructive surgery (**Table 1**): the Inventory of Potential Reconstructive Needs (IPRN) of Brou et al.[68] and a modified version by Fisher et al. (MIPRN)[57] were introduced in the pediatric burn population, and scored both functional (e.g., contractures) and cosmetic aspects. The MIPRN showed an inter-rater variability of 0.996[57]. Rea et al. also included pain and analgesia usage[58]. These scales help to systematically prioritize and evaluate options together with the care-giver, surgeon, and therapist, as well as facilitating or encouraging adoption of realistic expectations for the child or parent. Several patients discern improvement of their scars and seem to accept their scars over time, which may explain the finding that surgeons would like to operate more scars then the patients themselves[58, 62]. Therefore the patient's point should be central in the assessment of reconstructive needs[58, 62].

Discussion

Due to major advancements in burn treatment, the emphasis in modern burn care has shifted to 'quality of survival' (functional and esthetic) instead of 'survival' only. Since quality of survival mainly depends on the impact and disability of the residual scars, prevention, treatment, and assessment of burn scars have become increasingly important. The first scale to assess (burn) scars was described 30 years ago, but since then almost 20 different scar scales and modifications have been developed. None of them really stands out or is generally accepted, but the VSS and POSAS are probably most used in practice. Some general trends in scar assessment are nevertheless noted over time, especially the change in focus from only the opinion of the physician about the scar, to scoring the actual impact of the scar for the patient (e.g., psychologic impact, pain, itching, contractures.). All scales assess several variables, of which the visual aspect (e.g., color, thickness, and roughness) used to be the most important (**Table 2**). But as seen by the inter-rater variability reported in several studies, considerable variations can be seen between different assessors (and also between assessments by the same assessor), especially for the separate subscores. This means

that in general, it appears that a 'bad' hypertrophic scar will always be scored as such, but the actual severity might vary considerably among assessors.

Variable	Described as
Colour	- Vascularization: pale/white → dark red/purple - Pigmentation: hypo → hyperpigmented or combination
Thickness	- Raised or depressed?
Surface	- Smooth → irregular - Soft → palpable → hard - Supple → contractures
Border or contour	- Unclear → prominent
Itching an pain	- Visual analogue scales - Use of analgesics etc.
Localization and measuring of surface	- Measuring of width - Body charts - Photographic documentation
Surgical parameters	- Hatch and suture marks
Overall cosmetic and functional disfigurement	- Visual analogue scales - Mobility: range of motion, activity level - Need for reconstructive surgery

Table 2. The most important variables assessed by scar scales.

The opinion of the patient gradually became valued, and this seemed to deviate frequently from the opinion of the physician[58, 62]. For the patient, the presence of itching and pain is often more disturbing than the actual appearance, and therefore these symptoms have a major impact on the quality of life, as well as the major psychologic impact due to the appearance of the scars[12, 13, 15, 17-20]. Later on, the influence of the scar on the activity level and resulting disability were also scored, while other scores only focused on the need for reconstructive surgery.

Already in the late 1980s, standardized photographs were introduced to facilitate uniform scoring and improve reproducibility of the results. In the last decade, digital photography became widely available due to the low purchase price of digital equipment (computers and cameras), and the limited extra costs and workload related with the processing and storage of images. Pictures can also be helpful in clinical settings where patients may be followed by different physicians. Therefore, a digital camera is now a standard tool used in the follow-up in several medical specialties such as traumatology, dermatology (nevi follow-up, skin diseases), and of course reconstructive and aesthetic surgery. Although there appears to be a trend to restrict scar assessment in a clinical setting to photographs (since a digital camera is readily available), a scar scale still provides more information than a picture only (e.g., stiffness of the scar, pain, itching). Moreover, a scar scale can still be included more

easily in the electronic file of a patient, compared with the uploading of pictures during out-patient clinic or ward rounds. A scar scale is also useful for a complete assessment of all important characteristics of the scar, especially for novice therapists. The assessment by young therapists should consequently be as reliable as by experienced therapists. Beside this, the advantages of quality of life scores (assessing the psychologic impact and disability) have also been demonstrated, and are certainly a valuable addition to the scar scales. However, a more extensive scale, which combines a 'scar scale' and a quality of life scale, will increase the work-load for the physician, and therefore quality of life scales are allocated for the follow-up by the psychologist.

The key questions of this article were when you should use scar scales, and which one should be used, but there is no clear-cut answer. Cost effectiveness should therefore be balanced against clinical effectiveness: scar scales are low-cost (e.g., no need for training or expensive tools) and do not necessarily increase work load considerably, in contrast to most scar tools, which require experience and a lot of additional time. The few minutes it takes to fill in most scar scales are probably not even worth mentioning, although implementing a scar scales implies changing the daily routine.''

The clinical effectiveness of scar scales is less clear: most scales were developed on small, mostly Caucasian populations, and were never validated. The statistical evidence was usually poor (low inter-rater variability), showing low reproducibility. The choice of a scar scale or tool will mainly depend on the purpose of the scar evaluation. For clinical follow-up, a digital snapshot often provides a sufficient, easy, and fast documentation of the scar, especially if it can be easily included in the electronic file of the patient. Scar scales provide additional information about, for example, texture of the scar, itching, and pain, but take a couple of minutes more time, depending on the included number of characteristics. The POSAS scale will, for example, require more time than the VSS because the opinion of the patient is included but, on the other hand, it provides more detailed information about the impact of the scar. To obtain a more objective and accurate analysis of the scar, e.g., for scientific purposes, assessment by scar tools (e.g., colorimetry) is advisable, but more expensive. A uniform assessment of scars should nevertheless be promoted for research (same calibration etc.), and would enable noninvasive comparison of different burn wound and scar treatments (without scar biopsies). For medico-legal purposes, a quality of life scale might be more useful, since these scales reflect the 'impact' of the scar more adequately than the actual appearance of the scar as described by the scar scales.

A limitation of this study, as well as of most studies describing scars, is the focus on burn scars. Burn scars are studied most frequently since they are relatively common and may lead to considerable morbidity. Other scars are seldom as extensive, and therefore usually do not cause substantial physical and psychologic morbidity. Burn scars present some specific problems as well (which are rare among other types of scars), such as the irregular appearance and the number and extent of the scars. Therefore, it can be assumed that scar assessment might be less complex in other

types of scars, and that these scars also have less clinical and therapeutic consequences, although there are certainly exceptions such as in case of extensive oncologic resections. Consequently, more complex scales, which are adequate for burn scars, will probably suffice for other scars as well, but the reverse situation might be more difficult. Hence, studies which compare the different scar scales among broad populations with scars of different etiologies would be useful to compare the efficacy of the different scar scales in different fields of application.

To conclude, although scar scales are less objective than scar tools, they are a worthy addition to digital photography for clinical evaluation and follow-up of burn scars. However, scientific studies about the effectiveness of the different scar scales are scarce. Irrespective to scar assessment, a multidisciplinary approach remains essential in the follow-up of patients with excessive scarring, including psychologic therapy and physiotherapy.

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CHAPTER 7

BURN SCAR ASSESSMENT - A SYSTEMATIC REVIEW OF OBJECTIVE SCAR ASSESSMENT TOOLS

BURNS (2010)

N. BRUSSELAERS
A. PIRAYESH
H. HOEKSEMA
J. VERBELEN
S. BLOT
S. MONSTREY

Abstract

Purpose

All deep second and third degree burns are at risk to develop hypertrophic scars which can severely undermine the quality of survival. To assess the severity of scarring, several technical devices or tools have been introduced to evaluate one or more aspects of the scar, enabling comparison of different treatment protocols and allowing an objective follow-up. The objective of this study was to review which tools can be used in objective burn scar assessment.

Basic procedures

The Systematic literature search involving PubMed, the Web of Science (incl. Science Citation Index).

Main findings

51 articles with burn scar assessment as main topic were found. Several characteristics of the scar can be assessed, such as color, metric features and elasticity, but none of the available tools covers the whole aspect of the scar. Especially subjective factors such as pain and itching cannot be assessed with those tools, in spite of their great impact on the patient's quality of life.

Conclusions

Scar tools enable objective and reproducible evaluation of scars, which is essential for scientific studies and medico-legal purposes, and in selected cases for the clinical follow-up of an individual patient. Further studies to evaluate these tools on scars are nevertheless required.

Introduction

In the past decennia, major improvements in burn management have resulted in a substantially increased survival of severely burned patients[1-3]. Unfortunately, this has not always been paralleled with a similar increase in quality of life for these patients[4,5]. One of the major long-term problems in burn care is the formation of hypertrophic scars, which lead to aesthetical but also functional problems (e.g. contractures) and also cause a considerable psychological burden. Therefore, even the early burn treatment is guided and influenced by the risk of hypertrophic scar formation. The assessment of the natural healing potential is for example based on depth assessment, which also predicts the risk of abnormal scarring. The critical depth for excessive scar formation is in the deep dermis meaning that superficial burns should heal without leaving a scar, while deep burns always are 'at risk'[6-9]. Consequently, the longer the healing, the higher the risk of hypertrophic scarring[10]. The general rule in burn surgery is therefore to operate burns which will not heal within 2-3 weeks after the initial trauma[7,11,12].

To assess the severity of scarring, several scar scales have been developed over the last 30 years, of which the Vancouver Scar Scale and the POSAS scale (Patient & Observer Scar Assessment Scale) are the most widely used[13-16]. Scar scales include several variables such as color, extent and may even contain subjective factors such as pain and itching which are subject to major inter-patient variations. In general, scar scales are considered to be a subjective scoring system, because it is susceptible to important variation between different assessors (inter-assessor variation). To obtain a more objective evaluation of the scar, several devices or tools used in other medical specialties or even in the industry (e.g. assessment of textile color, elasticity of plastics) were introduced for the assessment of scars. These tools should provide a more objective and reliable evaluation of the scar, by a better reproducibility and lower inter-assessor variation.

In this overview we only focus on the scar tools, addressing the applied physical principles, and mentioning the most commonly described tools used for burn scar assessment.

Methods

Criteria for considering articles for inclusion

Articles dealing with non-invasive burn scar assessment with technical devices as a major topic were included. Scar scales without any technical analysis are excluded as well as histopathologic evaluations of scar biopsies. Articles comparing the influence of wound or scar treatments were also excluded.

Search methods

We conducted a systematic literature search involving PubMed and the Web of Science (which also contains major congress abstracts)[17,18]. The Cochrane Library did

not contain relevant articles. We searched PubMed from 1960 until February 2009 (date of search 18 February), using the Medical Subject Headings (MeSH) 'Burns' and 'Cicatrix' (**Figure 1**). This search retrieved 1974 articles, whereof only articles with those terms as major topic were included, and the MeSH 'burns' were not exploded, excluding articles about chemical, electrical, eye and sun burns and inhalation injury (n = 928). Limits were set to English, French and Dutch articles about human studies (n = 597). The MeSH terms 'Surgical flaps' and 'Neoplasms' excluded irrelevant articles dealing with flap surgery (n = 78) and cancer development in burn scars (n = 91). From the remaining 428 articles, 67 articles were selected based on title and abstract, of which 30 articles corresponded with the inclusion and exclusion criteria[19-48].

We also searched the Web of Science on the terms 'scar', 'cicatrix', 'burns' or 'burn', but this search was too wide, because, even after language selection, it led to almost 44,000 hits. Therefore, more strict combinations were performed ('burn', 'burns', 'thermal injury', 'cicatrix' and 'scar'), leading to six additional articles[15,49-53].

After searching reference lists and Science Citation Index of the relevant articles, 15 additional articles were included[54-68]. We finally selected 51 articles, including six reviews and editorials.

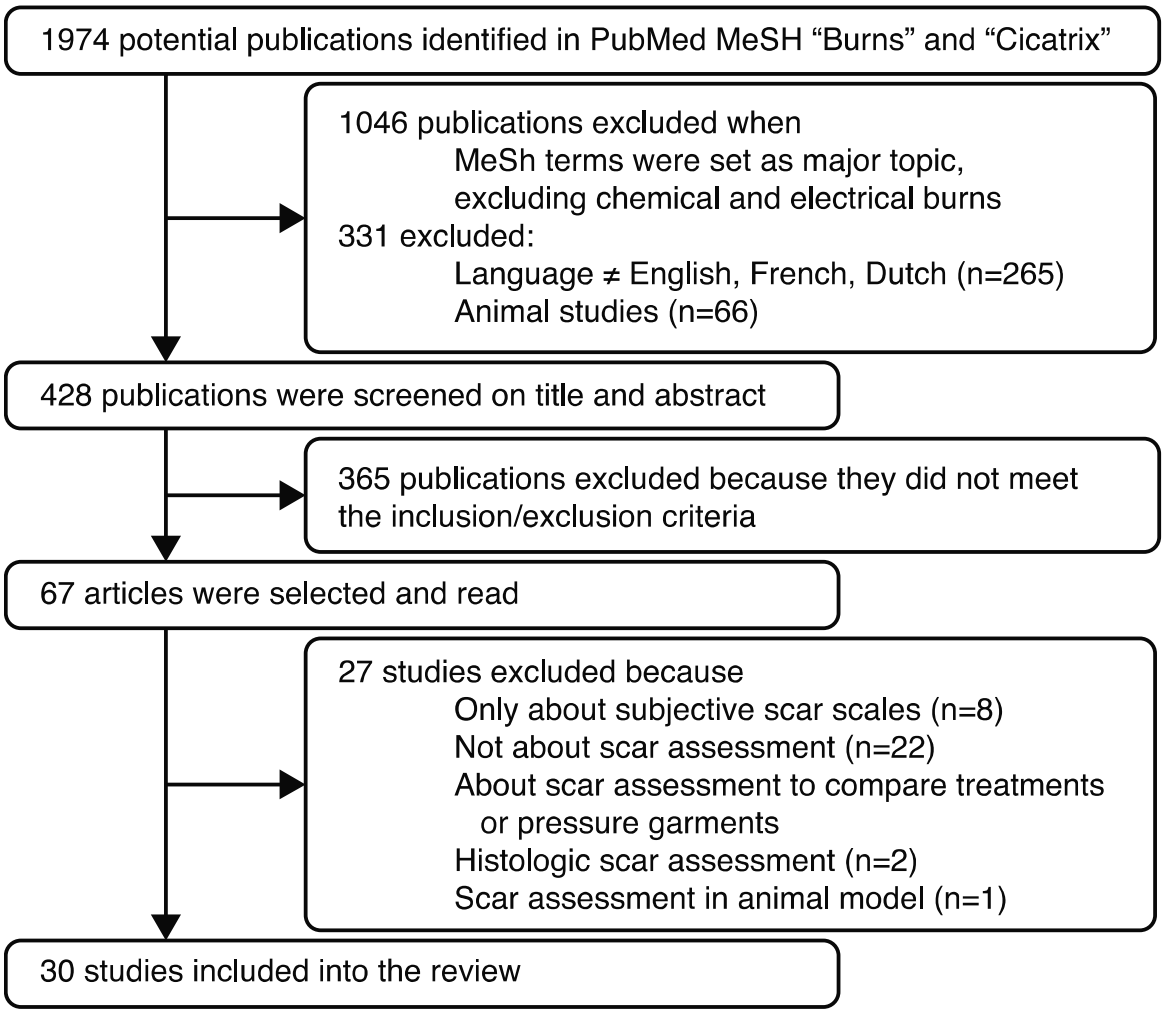

Figure 1. Flowchart of the systematic literature search (PubMed).

Quality assessment

Reproducibility of the assessments of these tools is evaluated (if described), and if possible the additional value of the device compared with visual assessment (e.g. with the scar scales) and/or other tools is discussed. The amount of articles (number of references) discussing a certain technique for scar assessment also reflects the relevance of implementing a similar device in clinical practice and its current popularity.

Results

Original articles, reviews and editorials dealing with burn scar assessment were retained. We chose to classify these tools into 4 groups based on the assessed variables: (A) color (vascularization, pigmentation), (B) metric variables (extent, height and volume), (C) biomechanical properties (e.g. elasticity, stiffness) and (D) physiologic changes (e.g. hydratation).

Colour evaluation

Color is probably the most complex characteristic of a scar and is mainly composed out of 3 components: the brown melanin pigment, the red oxyhemoglobin in the cutaneous vasculature (amount and oxygenation of blood vessels) and the yellow/ orange bile and carotene pigments[39]. The thickness of the skin layers, the reflection from the skin surface (texture) and the circumstances (e.g. temperature, light) also influence the color perception[38,39,69]. When using video or photographic images, these are strongly influenced by the settings of the camera (e.g. aperture, shutter-time) and the circumstances (e.g. light, temperature)[59].

Visual assessment is an effective but subjective way to evaluate color, with a considerable inter-observer variation[15]. Although the observer may distinguish thousands of colors, the human brain cannot reliably and accurately quantify the color or its intensity[15,31,33,42]. Moreover, memorizing colors is difficult, complicating the quality of scar color ratings for follow-up[33]. Therefore, several tools are developed to evaluate color in an objective and reproducible way, classified by the used principles: (1) reflection or absorption of light, (2) laser based methods and (3) computerized analysis of photographs.

Reflectance and absorption of light

It is not possible to obtain a perfect correlation between skin melanin or blood content and skin color[30]. Therefore, the optimal method to assess skin color is not histologic or chemical but spectrophotmetric[30,56,70,71]. Spectrometry is based on the reflectance and absorption of light and describes (i) the brightness and changes along the red-green and yellow-blue axis or (ii) the absorption of red and green light by melanin and hemoglobin respectively, resulting in the erythema and melanin index[30].

Different devices are used for color analysis of scars and skin diseases: (i) tristimulus colorimeters such as the Minolta Chromameter® (Konica), Labscan® (HunterLab) and the Micro Color (Dr. Lange GmbH), and the (ii) narrowband simple reflectance meters such as the DermaSpectrometer® (Cortex Technologies) and the Mexameter® (Courage&Khazaka)[20,27,52,71]. These tools assess the vascularity and pigmentation better than scar scales and enable immediate 'on-site' evaluation[20].

Laser based methods

The laser based methods assess the bloodflow and apply red or near-infrared wavelengths[33]. A considerably higher bloodflow is noted over immature burn scars, due to a higher vascularity. Structural changes may nevertheless interfere with perfusion measurements[33,73].

The Laser-Doppler Flowmeter (LDF) is used for the evaluation of cutaneous bloodflow to evaluate scar color[15,30,42,59,60,66,72]. It measures the flow over a small location, limiting its value for extended, heterogeneous surfaces[59]. This flowmeter is less sensitive than simple visual assessment of erythema and is therefore not recommended for color assessment[66]. The Laser-Doppler Imaging (LDI) is a laserbeam which is used to scan several points across a tissue surface, generating a 2D color coded image directly related to the bloodflow[19,31,33]. It is used for burn depth assessment[7,74-77] but can also be used for scar evaluation, with fast and reproducible results[19,31,33]. Another alternative is the Laser Speckle Imaging (LSPI) which uses digital image-processing techniques[31]. Moving red blood cells create dynamic interference patterns that change in time. The bloodflow maps are generated by coherent light reflected from stationary tissue producing a highly contrasted speckle pattern remaining static in time. LSPI allows for zooming in and increasing the resolution on a smaller field of view, in contrast to the LDI[31].

Computer analysis of colour

Even standardized photographing fails to compare scars objectively when analyzed by the human brain[30,66]. Therefore, several computer programs were developed to assess (digital) photographs[59,60]. At first, color photographs were converted into black and white (BW), because of its less complex electronic make-up[60]. The HSV-method analyzes three different aspects of color: the hue (dominant wave length e.g. red), the saturation (amount of white) and the value (amount of black), which are important in discriminating between colors, whereas in practice, differences in value mostly reflect varying levels of illumination[66,78-82]. The colors can also be represented as combinations of the amount of red, green and blue ('primary colors') (RGB model)[15,30,66], or by the proportions of cyan, magenta and yellow ('secondary colors') and black (CMYK model). These 3 color models are equivalent and conversion between them is simple[41]. A card carrying standard colors (e.g. Pantone®) is used to frame the scar so that every picture would include areas of known color properties[34,58]. Hereby, the influence of lightening conditions and camera settings can be subtracted, enabling an objective color evaluation[30,38,41,65,66].

Metric variables

Planimetry

Planimetry (or measuring surface area) is used to assess the extent of a scar and to detect contraction in time[15,30,41,65,66]. The main problem is that scar margins become more difficult to delineate during scar maturation. Tracing these margins on clear plastic film and photography are most commonly applied[66]. Photography is readily available, accurate and reliable (especially on flat or moderately curved surfaces), but standardized conditions are essential (distance, light, camera settings). Computer programs e.g. Image Tool® (C.C. Wilcox) can be used to determine the percentage of hypertrophic scars over the total scar area[30].

Height and volume assessment

Up to nine-fold increases in thickness have been described in scar tissue, but decreases can also be present[46]. Hypertrophy and atrophy are quantified by measuring scar thickness or volume. The height of a scar can be evaluated subjectively but inaccurately, since the portion of the scar below the surface is not included[44]. Some authors recommended histologic analysis of biopsied tissue (invasive technique), but skin biopsies may change in thickness when released from the tension and support provided in situ[30,66]. It can also be questioned if the biopsy site is representative[44]. Negative- positive moulds or replicas were used to make a 3D copy, accurately indicating height, extent and general appearance of a scar[46,66,67,83]. This technique can be combined with photographs and tonometric assessment, and is also useful for evaluating the roughness[46,60,66,84]. High frequency ultrasound (5-20MHz) tools such as the Dermascan® (Cortex Technology) provide reliable and accurate quantitative information on scar thickness[22,26,35,40,44,48]. It is very sensitive in the localization of scar tissues, distinguishing them from normal skin, and for assessment of thickness and delineation of the extent of the scar[40,44,65]. Therefore, portable devices e.g. TUPS (tissue ultrasound palpation system) were developed, facilitating clinical application. Although 3D ultrasound is available for clinical application, it is not widely used in scar evaluation because of its high costs[26]. MRI has been used for the evaluation of normal skin but has not yet been applied on scars[15].

Three dimensional techniques

Highly sophisticated, often expensive 3D methods became available for volume assessment, planimetry and analyzing roughness, including the use of full-body morphometric scanning, range scanners and 3D reconstructions (e.g. Vivid 900, Konica-Minolta and Vectra 3D imaging system, and Canfield Imaging Systems), but scientific studies assessing burn scars remain scarce[30,41,54,64,65]. Advantages are the fast and direct, non-contact measurement of the surface and volume of the scar, macro- and micro-topometry, high resolution, high precision, and ease of handling[60,85]. Range scanners project a light pattern onto a scene, which is

photographed by a regular camera[38]. If the skin surface is uneven, which is the case in hypertrophic scarring, the projected light pattern appears distorted, which enables inferring the depths of points in a scene[38,52].

Biomechanical properties

Elasticity or stiffness

Several mechanisms can be used for evaluation of elasticity or stiffness. The elasticity of the skin is the property to return to its original shape when the stress is removed which caused deformation (e.g. external forces). Stiffness is the resistance of an elastic body to deformation by an applied force and can be quantified easier than elasticity. These methods described here originate from dermatology (e.g. Cutometer®, Dermaflex® and Dermal torque meter®), ophthalmology (tonometers) and from industrial applications (durometer). These 'elastometers' can be classified by the applied biomechanical forces[66], which can be in a vertical direction: (i) suction or (ii) pressure; or horizontal: (iii) torsion or (iv) extension.

1. Suction methods: a controlled negative pressure is exerted over a small area of the scar, resulting in a skin deformation which is analyzed by a computer[35,37,38,51,52,86]. The Cutometer® (Courage&Khazaka) proved to be highly reliable and reproducible for burn scars except for the most severe scars[23,24,51,68,86], but the size of the tool can be considered impractical[30]. The Dermaflex® (Cortex Technology) is an alternative device with a larger diameter of the suction chamber (10 mm vs. 6 mm), but no scar assessment trials have been published yet[49,66].
2. Pressure methods or 'tonometers'[38,66] originate from measuring intra-ocular pressure[43,48] and hardness of metals and plastic[57,87] and calculate the power required to produce a certain deformity[43]. Several devices are developed and evaluated for skin elasticity measurement[87], of which several prototypes were tested on scars: cicatrometers[48], pneumatonometers[30,62], tonometers[25,28,43] and durometers[30,57]. They produce good results, but cannot be applied on scars above bone structures[66].
3. Torsion methods[49,66] such as the Dermal Torque Meter® (Dia-Stron Ltd.) measure the torsion force needed to deform the skin. Only one scar study has been published, which reports resemblance with measurements with the Cutometer®[49,66].
4. Extension methods or 'extensometers'[66] stretch the skin between two tabs to assess differences in extensibility or stiffness. This method has been described for scar evaluation[38,50,66], but scientific results are scarce[68].

Acoustic methods

Sound waves (5-8 kHz) are used to detect heterogeneity in the scar tissue, e.g. Shear Velocity Device, Reviscometer® (Courage&Khazaka)[36,66]. A higher velocity (or speed) of wave transmission indicates a more dense structure (less deep penetration

of the waves), correlating with a higher degree of stiffness, related with scar contraction[36]. These waves lie within the spectrum of normal hearing (which is 20Hz-20 kHz) and penetrate deeper in the skin than the ultrasound waves (5-20MHz), but both techniques have not been compared yet for scar evaluation[36].

Disability measuring

Because contractures primarily occur in joints, burn scars often compromise mobility. The mechanical impairment can be estimated by measuring the range of motion of a joint and is even included in some subjective scar assessment scales[88]. The range of motion can be measured with goniometers (Greek for 'measuring an angle')[66,89,90]. This term is used for simple plastic tools as well as computerized devices[91].

It is also recommended to measure the disability itself (coordination, strength, skin sensibility)[66], e.g. by assessing daily life activities e.g. hand function[66]. The faciometer® is an electronic device originally developed to assess the results after reconstructive surgery in cases of facial palsy. It consists of two calipers connected to a digital display, showing the actual distance between the calipers. Measurements of distances between specific stable and moving points are made at rest and after standardized maximal and submaximal (mimic) movements, enabling a 3D analysis[53]. This tool proved to be useful for objective description of results after surgery for facial burns[53].

Pathophysiologic disturbances of the scar

Transcutaneous oxygen tension

Scar maturity has been related to transcutaneous oxygen tension[15,52,55,61] which can nowadays be measured with electrodes on the skin (previously by subcutaneous needles)[61]. It is based on redox reactions occurring in the electrode modified by the inclusion of a heat source measuring the oxygen and the carbon dioxide that diffuses through the skin[92]. In hypertrophic scars the PO2 is lower than in healthy skin, but an increase is described which correlated with clinical improvement over 60 weeks of therapy[55,61]. This technique seems to be abandoned for scar assessment, but is still used to assess limb ischemia[92,93].

Transepidermal water loss and moisture content

The skin acts as a barrier against permeation of external substances, as well as the water evaporation from the internal living tissue[52]. The water content in the skin preserves the softness and smoothness of the skin surface[52], and this can be measured directly or by the transepidermal water loss (TEWL)[52,56,94-100]. TEWL is strongly related with the moisture content of skin and can be measured with open or closed (insensitivity to external air currents) chamber systems. The open systems (e.g. Dermalab TEWL module, Tewameter®, Courage&Khazaka) are the oldest and still

most widely used[52]. The advantages of the closed chamber systems (VapoMeter, Delphin Technologies) need to be determined[95].

Another method measures the hydratation of the skin surface (stratum corneum), which is directly proportional to retention of electrical charge, and can be measured by e.g. Skicon-2001 conductance meters (I.B.S. Co., Ltd), CorneoMeter® (Courage&Khazaka), and the DermaLab® (Cortex technology, also measures TEWL and elasticity)[52,56,99,101,102]. These techniques are popular in the cosmetic industry, but also useful for evaluation of contact dermatitis and burn scars[52,56,94]. Scar sites are dryer than control sites and seem to become dryer as they mature[52,56,94,100]. The effect of environmental factors such as humidity (sweating) should be avoided, and showering and topical products are not allowed hours before measurement[56,103].

Discussion

Because hypertrophic scars are one of the major long-term problems after severe burns, scar prevention, treatment and assessment are of utmost importance. However, scar assessment is still a neglected area in the burn care, and a consensus about the ideal scar scale or tool is still lacking, probably due to the scarce amount of scientific studies. Several tools are currently promoted for (burn) scar evaluation, but these tools are mainly developed and commercialized for dermatologic use or for the cosmetic industry. Consequently, reference material for scar tissue is usually lacking, and no trials have been performed to compare the different tools for scar evaluation. Nevertheless, for most devices, the evaluation of skin or scar tissue should always be compared with a reference area of the patient, e.g. the other arm, because skin properties may vary considerably depending on the location on the body. The most important characteristics of the scar which can be analyzed by scar tools are the color, the thickness, the stiffness and the measurement of transepidermal water loss. These can all be assessed by different biomechanical techniques (sometimes combined in one device), with various degrees of complexity. The test results are preferable directly registered or integrated automatically in the computer system.

The question may rise what the therapeutic consequences are of evaluating scars, if you already use all preventive measures currently available. Nowadays, it is still difficult to predict which burn wound will certainly result in hypertrophic scarring and therefore preventive measures such as pressure therapy, splinting and silicones have become routine practice for all deep, extended burns in most burn units. Yet, it is useful to have an objective method to evaluate the degree of maturation of a scar, because it enables early adjustment of the therapy by introduction of extra preventive measures or earlier treatment e.g. by corticoid injections.

There is no doubt that objective scar assessment by scar tools definitely has an additional value in scientific studies, because different scars can be described and analyzed in more detail and compared mathematically. Therefore tools are statistically superior to scar scales and pictures; however the number of assessed variables is more limited than in scar scales. The role of scar tools in the daily clinical practice is less clear, because the tools are often large and expensive and increased

workload, time and costs. For this reason, scar scales are considered more cost-effective and can also be used more easily in clinical practice (optimally combined with digital photographs). However, the scales are less objective than the tools due to the large inter- and intra-observer variation. In the (near) future, scar tools should become more accurate and reproducible than scales, and should detect derailment of the scar maturation earlier on, enabling earlier adjustment of therapy. Yet, at this stage, it is not possible to point out one ideal tool, and the optimal balance between accuracy, clinical and cost-economic applicability is still not reached within one single scar tool. Which tools will become more important will also be guided by the insight into the pathophysiology of scar formation. Recent studies report for example the major impact of the moisture content in scar maturation[104]. Further comparative clinical trials are required to compare the reproducibility and accuracy of the scar tools.

To conclude, advances in technology resulted in several new promising techniques, but more scientific studies are needed before these scar tools can be implemented in the scientific and routine burn assessment. Besides scar tools, which can only assess a limited number of characteristics, an additional clinical evaluation will remain necessary, preferably by applying digital photography and a scar scale including the patient's perception of their scar (including pain, itching…) and the impact of the scar on the quality of life.

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CHAPTER 8

GLYADERM DERMAL SUBSTITUTE - CLINICAL APPLICATION AND LONG-TERM RESULTS IN 55 PATIENTS

BURNS (2015)

A. PIRAYESH
H. HOEKSEMA
C. RICHTERS
J. VERBELEN
S. MONSTREY

Abstract

Introduction

Glycerol preserved acellular dermis (Glyaderm®) consists of collagen and elastin fibers and is the first non-profit dermal substitute derived from glycerol-preserved, human allogeneic skin. It is indicated for bi-layered skin reconstruction of full thickness wounds. Methods: A protocol for clinical application and optimal interval before autografting with split thickness skin graft (STSG) was developed in a pilot study. A phase III randomized, controlled, paired, intra-individual study compared full thickness defects engrafted with Glyaderm® and STSG versus STSG alone. Outcome measures included percentage of Glyaderm® take, STSG take, and scar quality assessment.

Results

Pilot study (27 patients): Mean take rates equalled 91.55% for Glyaderm® and 96.67% for STSG. The optimal autografting interval was 6 days (±1 day).

Randomized trial (28 patients): Mean Glyaderm® take rate was 88.17%. STSG take rates were comparable for both research groups (p=0.588). One year after wound closure, Glyaderm® + STSG was significantly more elastic (p = 0.003) than STSG alone. Blinded observers scored Glyaderm® treated wounds better in terms of scar quality.

Discussion

The efficacy of Glyaderm® as a suitable dermal substitute for full thickness wounds is attested. Currently a procedure for simultaneous application of Glyaderm® and STSG is adopted, allowing for further widespread use of Glyaderm®.

Introduction

Dermal substitution has become an integral part of surgical burn care and many commercial dermal equivalents have emerged on the market since the introduction of Integra® dermal substitute (Integra LifeSciences Corporation) some two decades ago[1-3].

We extensively reported on the various cellular, acellular, temporary and permanent skin replacements available for burns and full thickness defects in a previous publication[4].

Glycerol preserved acellular dermis (Glyaderm® - Euro Skin Bank, Beverwijk, The Netherlands) is the first non-profit dermal substitute derived from glycerol preserved, human allogeneic skin[4-6]. Glycerol preserved allogeneic skin (GPA) is routinely utilized as a temporary biologic dressing on partial thickness burns and as a means of wound bed preparation on excised burns. Allograft coverage prevents dehydration and infection of the wound and stimulates granulation formation to prepare the wound for closure with autologous skin[5,6]. Allografts contain donor cells, which are ultimately rejected and can therefore only be used as temporary wound coverage. Glyaderm®, which is decellularized by treatment with sodium hydroxide (NaOH), can be used to replace lost dermis in full thickness wounds serving as a dermal substitute. Glyaderm® consists of a collagen and elastin fiber network with native collagen and can ensure a bilayered skin restoration in combination with a thin autologous split skin graft. It is intended to be cost-effective and easy to use for widespread application in full thickness wounds such as full thickness burns. Glyaderm® is placed in a wound bed prepared with allografts, after which, a thin autologous split thickness skin graft (STSG) will close the wound following Glyaderm® ingrowth. Animal studies showed favourable results in terms of tissue integration and wound contraction and scar quality[6].

We first initiated a phase I pilot study to elucidate the most practical protocol for Glyaderm® application and to further investigate the scope of use of the dermal matrix in the clinical setting.

The second study was a phase III randomized, controlled, paired, intra-individual comparison of full thickness skin defects engrafted with Glyaderm® and STSG versus STSG alone.

Materials and methods

Enrolment

Between September 2005 and October 2010 27 patients were recruited for the pilot study and 28 patients met the criteria for inclusion in the randomized controlled, paired, intraindividual trial. Study protocols were approved by the Ghent University Hospital Ethics Committee. Glyaderm® was produced and provided by Euro Skin Bank, Beverwijk, The Netherlands. The preparation steps of Glyaderm® have been described previously[6].

Phase I pilot study

The pilot study was initially performed to assess the scope of clinical applications of Glyaderm® as a dermal substitute and to optimize usage protocol. Patients with full thickness burns, but also other full thickness skin defects were considered eligible for this study.

All burn wounds that were not clearly full thickness on clinical assessment were treated during the first 48 h with an enzyme alginogel (Flaminal® Forte-Flen Pharma)[7] and covered with a paraffin gauze dressing (Jelonet® - Smith & Nephew). Flaminal® Forte combined with Jelonet® ensured maintenance of a moist wound environment[7] for the first 48 h prior to assessment by laser Doppler imaging (LDI). This is the standard treatment for all burns admitted to the Ghent Burn Center.

In our burn center we use the moorLDI2-BI imager (Moor Instruments Ltd., Axminster, UK) to objectively determine the healing potential of the burn[8]. LDI is now becoming a standard of care for early diagnosis of healing potential, which is a main determinant of subsequent treatment policy. In clinical trials LDI ensures exact comparison between two burns without depth difference bias.

In this study, besides clinical observation, LDI was also intended to monitor the rate of vascularization into the dermal substitute and thereby to delineate the optimal time between the application of Glyaderm® and the final coverage with an autologous STSG. Ingrowth of blood vessels into Glyaderm®, resulting in increased blood flow through the dermal substitute, was assessed by means of LDI at day 1, 3, 5 after the application of Glyaderm® to the wound. An increase in flux values over the measurement period was interpreted as increased blood vessel ingrowth. Biopsies were harvested before autografting to support this hypothesis. In order to visualize blood vessel ingrowth into Glyaderm® the sections taken from the biopsies were coloured with antibodies against alfa-smooth muscle actin (ASMA) in order to demonstrate the presence of myofibroblasts and pericytes, which are supporting cells for blood vessels.

Efficacy of the protective open pore structure polyamide dressing (Surfasoft® - MediProf) and finally the coverage with a 10% povidone iodine (PVP-I) gel (iso-Betadine® Gel - MedaPharma Belgium) in combination with Jelonet® was tested.

Outcome measures were percentage of Glyaderm® take and percentage of STSG take.

Patients were invited for a long-term follow-up after complete scar maturation. The long-term scar assessment included objective measurement of elasticity with the DermaLab® (Cortex Technology, Denmark) and measurement of scar erythema and pigmentation with the DermaSpectrometer® (Cortex Technology, Denmark), as well subjective scar evaluation by means of the adapted Vancouver Scar Scale (aVSS) and the Patient and Observer Scar Assessment Scale (POSAS). The aVSS, besides scar colour, pigmentation, pliability and scar height also takes into account scar itching and the presence of defects.

In 4 patients biopsies were taken at 1 month and sent for histological analysis. Biopsies were fixed in 4% formalin and were further processed into paraffin. Sections

were prepared and stained with Haematoxilin-Eosin and Elastica von Giesson to study the presence of Glyaderm®.

Phase III study

Study design

This was a randomized, controlled, paired, intra-individual comparison of full thickness skin defects engrafted with Glyaderm® and STSG (experimental treatment) versus STSG alone (conventional treatment).

Study objective

Primary outcome measure was comparison of autograft survival at one week between full thickness defects treated with Glyaderm® plus STSG versus STSG alone.

Secondary outcome measures were the functional and cosmetic outcome of skin restoration of full thickness defects treated with Glyaderm® plus STSG versus STSG alone 1, 3, 6 and 12 months post wound closure.

Patient selection

Patients up to 80 years of age with full thickness burns or full thickness lower arm defects after free flap harvesting were considered eligible. Burn wounds had to be either clearly full thickness burns as clinically assessed by two plastic surgeons, or flux values measured by LDI had to be below 200, corresponding with a healing time clearly longer than 21 days. Eligible patients with the possibility to follow the complete treatment schedule were consented for the trial.

Patients with one or more serious medical conditions that, in the opinion of the investigator, made the patient an inappropriate candidate for the study, or any condition that seriously compromised the patient's ability to complete this study, were excluded. Patients with TBSA of over 40% and patients who had participated in another study utilizing an investigational drug within 30 days prior to study inclusion were also excluded.

Randomization

The experimental and conventional treatments were confined to anatomically related areas to allow a paired, intraindividual comparison. Preferably a right/left comparison was made; if not feasible, a superior/inferior or medial/lateral comparison within a wound surface area was performed.

To exclude any bias due to selection of the surgeon or the researcher, investigators received pre-sealed envelopes containing individual patient's treatment assignments according to a predetermined scheme randomizing the experimental treatment.

Randomization was performed in the operation theatre after the plastic surgeons had removed the allografts used for wound bed preparation and assessed the wound to be ready for STSG application. Usually this would be at the second operation, unless further wound bed preparation with allografts was necessary at that stage.

*Surgical regimen (**Table 1**)*

The first operation consisted of either full thickness removal of the burn scar performed as soon as possible after burn depth diagnosis, or the harvesting of the free radial forearm flap resulting in an almost circumferential (16 cm x 13 cm) defect.

	Glyaderm® + STSG (experimental treatment)	STSG alone (conventional treatment)	
Wound bed preparation	1st operation: Allograft	1st operation: Allograft	Wound bed preparation
Dermal substitute	2nd operation: Glyaderm®	2nd operation: Allograft	
Autografting	3rd operation: STSG	3rd operation: STSG	Autografting

Table 1. Phase III randomized trial patient treatment scheme

In both cases this was followed by application of glycerol preserved allografts meshed 1:2 for wound bed preparation.

The second operation was performed 5-10 days after the first operation and the surgery to be performed depended upon the quality of wound bed preparation with the allografts.

If wound bed preparation was not satisfactory, allograft application would be repeated. If wound bed preparation was satisfactory the experimental (Glyaderm® + STSG) and conventional (STSG) treatments were confined to anatomically related areas to allow a paired, intra-individual comparison according to the randomization scheme.

After removal of the allografts and scrubbing with a PVP-I 10% solution (iso-Betadine® Dermicum - MedaPharma Belgium) and saline, and hemostasis with adrenaline soaked gauzes, the wounds were treated with sutured or stapled application of Glyaderm®, perforated 1:1, on the treatment side and renewed application of allograft on the conventional treatment side. Both wounds were covered with Surfasoft®.

Final operation, also performed 5-7 days after treatment confinement, as guided by clinical assessment and supported by LDI, consisted of the removal of the allografts at the conventional side and gentle scrubbing of the Glyaderm® dermal matrix and the application of a STSG (0.010 in) on top of both study treatment areas. Mesh ratio was always similar for the experimental side as well as for the conventional treatment side. Autografts were covered with Surfasoft®.

Wound treatment regimen

All burn wounds that were not clearly full thickness on initial clinical assessment were treated once daily during the first 48 h with iso-Betadine® Dermicum for decontamination followed by application of Flaminal® Forte covered with a Jelonet® dressing and a dry sterile gauze dressing. Clearly full thickness burns were treated with cerium nitrate-silver sulphadiazine (Flammacerium® - Sinclair Pharmaceuticals Ltd.) until the first operation.

Allografts were covered daily with iso-Betadine® gel and® until the next operation. The same applies to Glyaderm®.

Autografts were dressed with Jelonet®, iso-Betadine® gel and a covering dry sterile gauze dressing until day one post application after which the wounds were dressed daily with, iso-Betadine® Dermicum soaked gauzes, Jelonet® and dry sterile gauze until removal of the Surfasoft® layer at day 6-7.

Donor sites were dressed with Hydrofiber® silver dressings.

Study assessments

All data were recorded in a purpose designed database.

Baseline research group characteristics

Patient demographics were recorded at study inclusion. Patient gender, age, burn cause, total body surface area (TBSA) that was burned in %, burn body location, TBSA represented by the target wounds in % were noted.

Wound evaluation

Clinical wound assessments were conducted twice weekly from inclusion to full wound closure. Wounds were photographed, if possible, the day of, or after admission and also the day of LDI and thereafter twice weekly and at every surgical procedure.

Wound swabs were harvested for semi-quantitative and qualitative microbiological investigation on admission, on the day of LDI and then repeatedly on a weekly basis from the region of interest as well as other burn areas according to a standard microbiology swab procurement regimen which exists as an integral part of the Ghent Burn Center wound care policy.

Take rates

Glyaderm® was evaluated with LDI at postoperative day 1, 3 and 5 for vascular ingrowth. Glyaderm® take rates were scored at day 6-7 post Glyaderm® application, during the autograft procedure. STSG take rates were scored at day 6-7 post autograft application and after Surfasoft® removal.

Treatment after wound closure

Pressure garments and silicones

Scar treatment was the same for both groups and consisted of custom made pressure garments and/or silicone garments.
There was an individual and especially adapted schedule worked out for every patient, regarding the silicone pressure garments.

Hydration of the scar

Hydration of the dry skin is necessary at least three times a day. All patients were using the same product Alhydran® (BAP-Medical)[9] during the complete follow-up period of 1 year.

Follow-up assessments

At regular follow-up of 1, 3, 6 and 12 months objective and subjective scar assessment was performed. Objective evaluation of elasticity was performed using the DermaLab®. For colour and pigmentation assessment of the scar, the DermaSpectrometer® was used.
For subjective measurements of quality of scar formation as for example the degree of hypertrophic scarring the aVSS as well as a subjective 5 Point Contour Scale, grading from severe contour deformity to normal anatomical contour, were used.

Statistical analysis

Statistical analysis was performed with SPSS 21.0 for Windows. Besides descriptive statistics, non-parametric statistical analysis of the groups was performed using Mann- Whitney U-test. Statistical significance was declared if $p \leq 0.05$.

Results phase I pilot study

Baseline group characteristics

Twenty seven patients, with a mean age of 32.30 years (±21.02), were recruited for the pilot study (**Table 2,3**). In one patient who received Glyaderm® after excision of a giant naevus, Glyaderm® was lost due to infection with pseudomonas aeruginosa. After removal of the Glyaderm®, control of infection and renewed wound bed preparation, the wound was re-grafted with Glyaderm® and STSG with full take. In 3 patients with a full thickness skin defect after radial forearm flap harvest and immediate application of Glyaderm®, there was no ingrowth of Glyaderm®. The protocol was changed to application of allografts to allow adequate wound bed preparation prior to application of Glyaderm®. After this change the Glyaderm® ingrowth in patients with radial forearm flap defects was satisfactory.

Pat no	Gender	Age (years)	Etiology	Localization	LDI
1	Male	33	Burn wound	Faceright	No
2	Female	32	Burn wound	Armright	No
3	Male	47	Burn wound	Handright	Yes
4	Female	74	Burn wound	Breastright	No
5	Female	29	Burn wound	Thorax/abdomen	Yes
6	Male	3	Giant naevus	Lowerleg right	No
7	Male	1	Burn wound	Handleft & right	Yes
8	Female	56	Burn wound	Neck	Yes
9	Male	20	Burn wound	Handright	Yes
10	Female	6	Burn wound	Armright	Yes
11	Male	34	Deglovement	Footright	Yes
12	Male	2	Giant naevus	Upperleg right	Yes
13	Female	58	Skin tear	Lowerleg right	Yes
14	Female	8	Burn wound	Armright	Yes
15	Male	2	Burn wound	Armleft	Yes
16	Female	47	Burn wound	Neck	Yes
17	Male	24	Radial forearm flap	Forearm left	Yes
18	Male	25	Radial forearm flap	Forearm left	Yes
19	Male	40	Radial forearm flap	Forearm left	Yes
20	Female	54	Burn wound	Face	Yes
21	Female	51	Fasciotomy	Lowerleg left	No
22	Female	60	Burn wound	Upperleg left	Yes
23	Female	51	Burn wound	Upperarm left	Yes
24	Male	28	Burn wound	Upperarm right	Yes
25	Male	27	Radial forearm flap	Forearm left, thigh left	Yes
26	Male	10	Burn wound	Thorax/abdomen	Yes
27	Male	50	Radial forearm flap	Forearm left	Yes

Table 2. Patients Pilot Study.

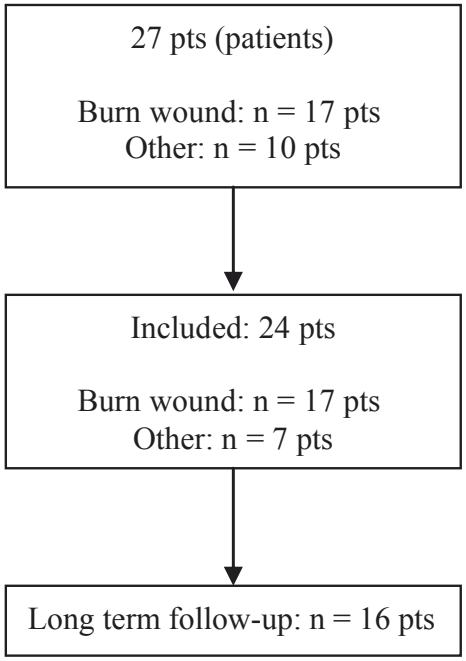

Table 3. Phase I pilot study patient enrolment

Take rates

Mean Glyaderm® take rate in the patients with Glyaderm® ingrowth was 91.55% (±14.59) and 75% of those patients had a Glyaderm® take rate of 95% or higher. Mean STSG take rate after Glyaderm® ingrowth was 96.67% (±4.75).

LDI demonstrated enhanced vascularization from day 1 to day 7, corresponding with both ASMA stained sections from biopsies (**Figure 1**), harvested before autografting, and clinical observation of the dermal substitute starting at day of Glyaderm® application until day of autografting. The colour coded map on the computer, created by the measured flux values, allowed us to delineate the optimal engraftment interval. The optimal time before application of a STSG on top of the Glyaderm® was 6 days with a 1 day standard deviation as shown in **Figure 2**.

All patients responded well to a dressing regimen of Surfasoft®) for protection of the Glyaderm® combined with iso-Betadine® Gel and Jelonet® in terms of bacterial control and prevention from dehydration and desiccation of the Glyaderm®.

Histological analysis with Elastica von Giesson staining, of the biopsies taken at 1 month post wound healing, confirmed the presence of a native and vascularized collagen-elastin matrix embedded between the epidermis and the subcutaneous layer, thus recreating a neodermis as shown in **Figure 3**.

Long-term follow-up

In total 16 patients participated in the long-term follow-up after Glyaderm® scar maturation (**Table 4**).

Variable	Statistical analysis	*p*-Value	Advantage
Elasticity (long term follow up) Glyaderm® + STSG vs normal skin	Mann-Whitney test	0.319	Glyaderm® + STSG compares to normal skin
Erythema (long term follow up) Glyaderm® + STSG vs normal skin	Mann-Whitney test	0.052	Glyaderm® + STSG compares to normal skin
Pigmentation (long term follow up) Glyaderm® + STSG vs normal skin	Mann-Whitney test	0.120	Glyaderm® + STSG compares to normal skin

Table 4. Phase I pilot study overview of statistical results (long-term follow up).

Elasticity measurements with the DermaLab® resulted in an average young modulus of 8.51 (±4.12) for Glyaderm® + STSG and 6.77 (±3.78) for normal skin. Statistics using the Mann-Whitney test demonstrated that, within this group of 16 patients, elasticity of Glyaderm® + STSG is not significantly different from the elasticity of normal skin (p = 0.319).

DermaSpectrometer® measurements for erythema were on average 15.21 (±5.31) for Glyaderm® + STSG and 11.66 (±3.14) for normal skin. Erythema measured in Glyaderm® did not differ significantly from erythema measured in normal skin (Mann-Whitney test, p = 0.052).

DermaSpectrometer® measurements for pigmentation were on average 31.69 (±4.67) for Glyaderm® + STSG and 33.34 (±2.90) for normal skin. Pigmentation measured in Glyaderm® did not differ significantly from pigmentation measured in normal skin (Mann-Whitney test, p = 0.120).

POSAS score for general impression of the Glyaderm® + STSG was on average 4.25 (±1.81) for the investigators and 3.77 (±2.62) for the patients. The POSAS score varies between 1 and 10 with 1 meaning the scar equals normal skin and 10 equalling the worst imaginable scar. From a statistical point of view there was no difference between the scores of investigators and patients (Mann-Whitney test, p = 0.288). Adapted Vancouver Scar Scale equalled 3.81 (±2.26) on average, where the values for aVSS can vary between 0 (best score) and 18 (worst score).

In the absence of statistically significant differences between Glyaderm® + STSG and normal skin we therefore concluded that long-term results of the phase I pilot study proved Glyaderm® to be a suitable dermal matrix for full thickness burns and large soft tissue defects as also illustrated in **Figures 4-6**.

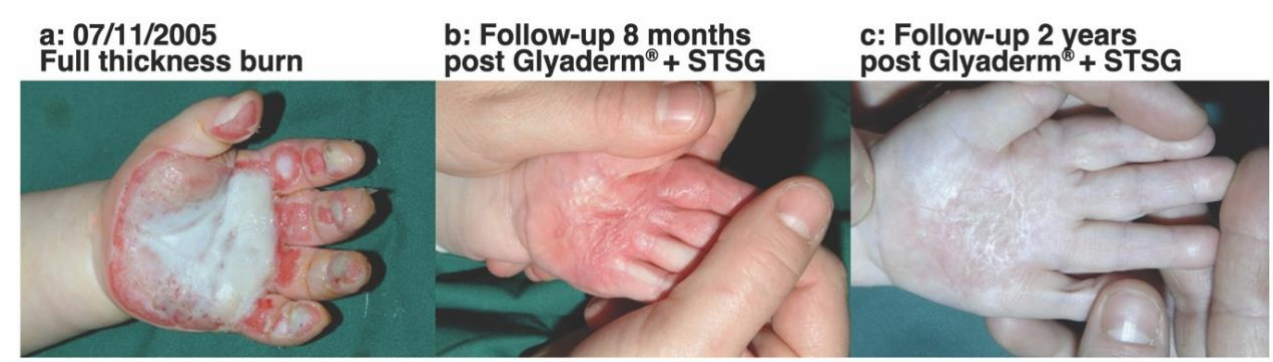

Figure 4. Full thickness burn in a 1-year-old boy.

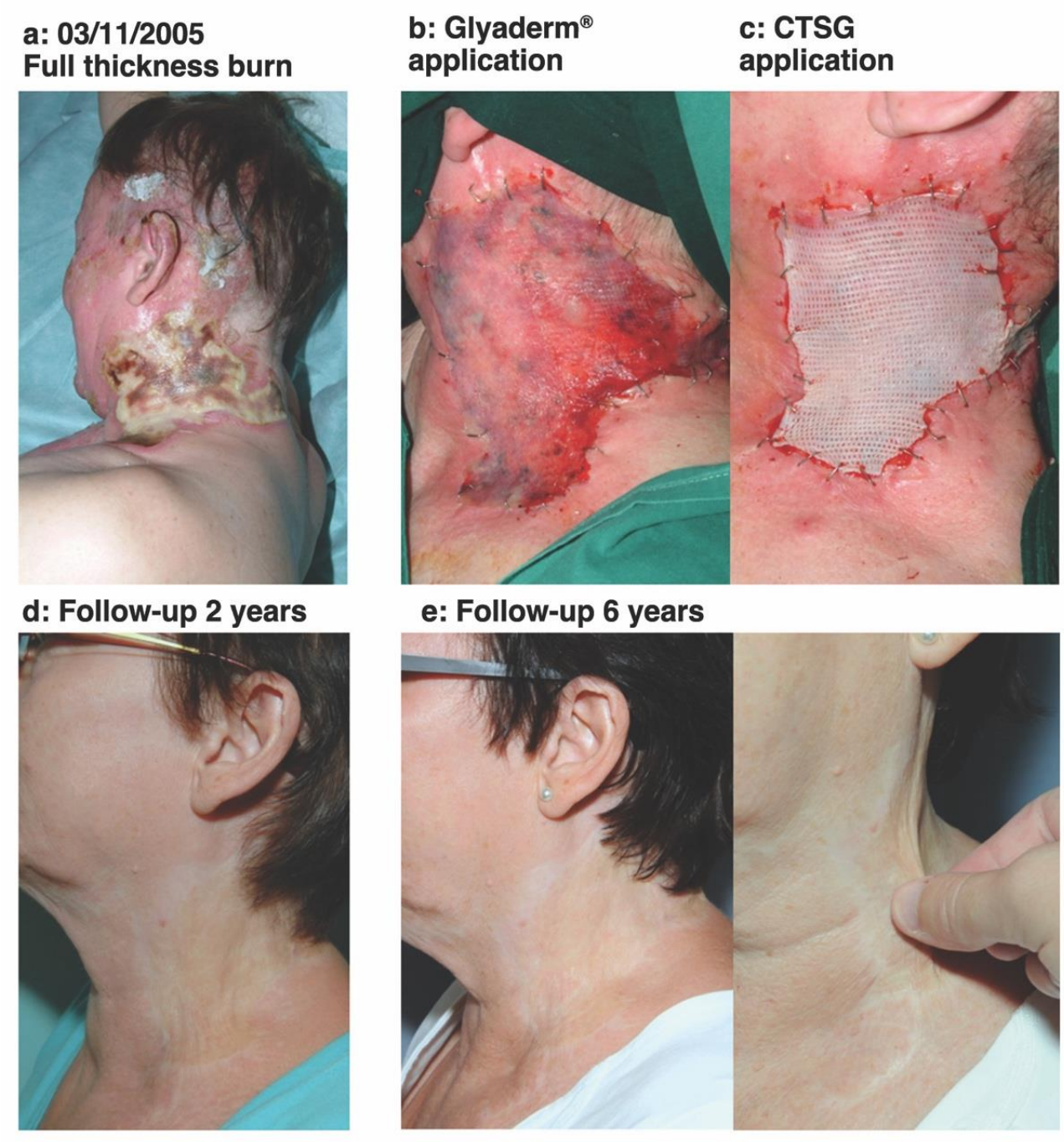

Figure 5. Full thickness burn in a 54-year-old woman.

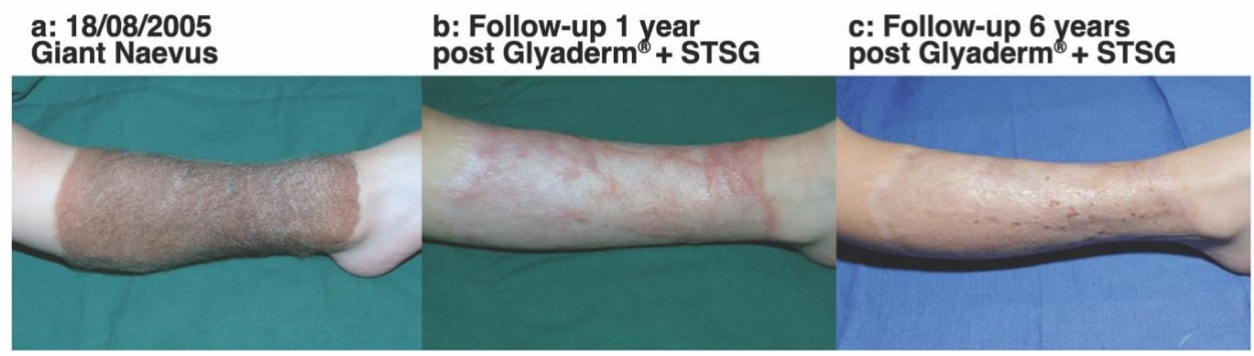

Figure 4. Giant Naevus in a 4-year-old boy.

Results phase III randomized trial

Baseline group characteristics

Thirty patients (34 sites) were eligible for inclusion in the study (**Table 5**). Two patients (two sites) were excluded prior to the Glyaderm® procedure. Twenty-eight patients with a mean age of 33.07 years (±10.35) and representing 32 sites met the inclusion criteria and were included in the study.

There were 9 patients with full thickness burns (13 sites) and 19 patients (19 sites) with full thickness defects after radial forearm flap harvest (**Figure 7,8**). Two sites (one in each group) were lost during the procedure due to no Glyaderm® ingrowth. Subsequent regrafting with Glyaderm® and skin graft showed good take but these were excluded from the study.

*Primary outcome measures (**Table 6**)*

Mean wound surface area of the wounds treated with Glyaderm® + STSG was 186.84 cm^2 (±165.20) and mean wound surface area of the wounds treated with STSG alone was 184.33 cm^2 (±175.87). Both procedures, as compared in this study, were comparable for treated wound surface area (Mann-Whitney test, p = 0.536) (**Table 6**).

Mean Glyaderm® take rate in the included patients was 88.17% (±18.34). Mean STSG take rate after Glyaderm® ingrowth was 92.47% (±23.19). STSG take rate in the wounds not treated with Glyaderm® was 97.68% (±4.99). The take rates of STSG in the STSG + Glyaderm® group were not significantly different from the STSG take rates in the group with a STSG alone (Mann-Whitney test, p = 0.588). Non-parametric statistical analysis in the subgroups based on wound etiology also resulted in comparable STSG take rates for burn wounds (Mann-Whitney test, p = 0.671) and for full thickness skin defects after radial forearm flap harvesting (Mann-Whitney test, p = 0.845).

*Secondary outcome measures (**Table 6**)*

On average, elasticity (Young modulus) measured 1 month after wound healing was 8.81 (±1.50) for Glyaderm® + STSG and 10.31 (±0.84) for STSG alone. 12 months after wound healing the Young modulus values averaged 8.89 (±1.10) for Glyaderm® + STSG and 9.29 (±0.99) for STSG alone. Comparing the DermaLab® measurements of "Glyaderm® + STSG" versus "STSG alone" statistics indicate that: "Glyaderm® + STSG" has significantly more elasticity when compared to "STSG alone" 1 month (Mann-Whitney test, $p = 0.001$) and 12 months (Mann-Whitney test, $p = 0.003$) after wound closure.

One year after wound closure we measured a mean Young modulus of 6.71 (±0.16) on normal skin which was significantly more elastic than both Glyaderm® +STSG(Mann-Whitney test, $p < 0.0001$) and STSG alone (Mann-Whitney test, $p < 0.0001$).

Variable	Statistical analysis	*p*-Value	Advantage
Mean wound surface area treated Glyaderm® + STSG vs STSG alone	Mann-Whitney test	0.536	Glyaderm® + STSG compares to STSG alone
Mean STSG take rate (%) Glyaderm® + STSG vs STSG alone	Mann-Whitney test	0.588	Glyaderm® + STSG compares to STSG alone
Elasticity (1 month after wound closure) Glyaderm® + STSG vs STSG alone	Mann-Whitney test	**0.001**	Glyaderm® + STSG
Elasticity (1 year after wound closure) Glyaderm® + STSG vs STSG alone	Mann-Whitney test	**0.003**	Glyaderm® + STSG
Elasticity (1 year after wound closure) Glyaderm® + STSG vs normal skin	Mann-Whitney test	**<0.0001**	Normal skin
Elasticity (1 year after wound closure) STSG alone vs normal skin	Mann-Whitney test	**<0.0001**	Normal skin
Erythema (1 month after wound closure) Glyaderm® + STSG vs STSG alone	Mann-Whitney test	0.072	Glyaderm® + STSG compares to STSG alone
Erythema (1 year after wound closure) Glyaderm® + STSG vs STSG alone	Mann-Whitney test	0.786	Glyaderm® + STSG compares to STSG alone
Pigmentation (1 month after wound closure) Glyaderm® + STSG vs STSG alone	Mann-Whitney test	0.581	Glyaderm® + STSG compares to STSG alone
Pigmentation (1 year after wound closure) Glyaderm® + STSG vs STSG alone	Mann-Whitney test	0.828	Glyaderm® + STSG compares to STSG alone
Adapted Vancouver scar scale (1 year after wound closure) Glyaderm® + STSG vs STSG alone	Mann-Whitney test	0.682	Glyaderm® + STSG compares to STSG alone

Table 6. Phase III randomized trial overview of statistical results. The bold *p*-values indicate statistical significance.

Measurements with the DermaSpectrometer® for erythema and pigmentation performed at 1 month and 12 months after wound closure did not result in statistically significant differences between "Glyaderm® + STSG" and "STSG alone".

When looking at the aVSS at 12 months, with a mean score of 3.27 (±2.76) for Glyaderm® + STSG and 4.73 (±2.01) for STSG alone, scoring is on average better for Glyaderm® although there is no significant difference from a statistical point of view (Mann-Whitney test, p = 0.682). aVSS scores for Glyaderm® noted in this study were comparable to aVSS scores for Glyaderm® observed in the pilot study. Independent blinded expert observers were asked to designate which of the intra-individual compared areas, according to their personal opinion, demonstrated best scar quality. According to these blinded expert observers best scar quality is mainly observed in Glyaderm® treated wounds (82%) as shown in **Figure 9**.

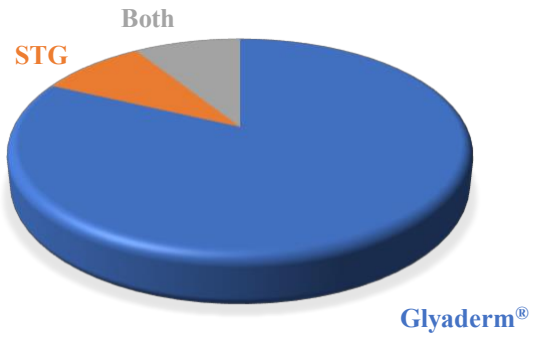

Figure 9. Best scar quality as subjectively attributed by independent blinded expert observers (phase III randomized trial).

Discussion

Excessive scar formation accounts for major morbidity and a continuing challenge in burn treatment[10]. Elasticity, flexibility, and strength of the normal dermis is compromised in scar tissue which can limit movement, causes pain, and is cosmetically undesirable[11,12]. The pivotal role of an adequate amount of dermis in surgical skin resurfacing is being increasingly understood and embraced[4]. The emphasis in surgical burn care has shifted from pure survival to quality of life after survival with increased interest in improvement of functional and aesthetic scar outcomes. Dermal substitution is becoming more and more a standard procedure in surgical burn reconstruction. Dermal substitutes are also being used for bi-layered skin resurfacing after trauma or (oncological) resections and in the field of breast reconstruction and hernia repair[13,14].

Elastin is historically underrepresented in commercial dermal substitutes, yet it serves a fundamental role in skin structure and function. The dermal elastic network determines skin resilience, texture, and quality but is poorly regenerated following burn[15]. In addition to its structural and mechanical functions, elastin has inherent cell signalling properties that promote a diverse range of cellular responses including chemotaxis, cell attachment, proliferation, and differentiation. Matrix elasticity and regeneration of the elastic fiber system is important for the development of functional dermal substitutes[15].

Collagen has been used in most dermal substitutes as it makes up the largest portion of the dermis, is biologically tolerated, and has well-defined structural, physical, and biological properties. One of the earliest and still most widely used commercial collagen-based dermal substitutes is Integra®[1-3]. It consists of a porous dermal layer made from bovine collagen and chondroitin-6-sulfate and a temporary silicone layer that acts as a barrier between the body and the environment. The silicone layer is replaced with a thin skin autograft following the substitute vascularization. During the wound healing process, bovine collagen is degraded and replaced by native collagen deposited by host fibroblasts. Collagen-based scaffolds currently dominate the dermal substitute field but are restricted by their lack of elasticity and impaired by scaffold contraction during repair[16,17]. Scaffold elasticity and regeneration of the elastic fiber system are now recognized as integral to the development of functional dermal substitutes[18-23]. The presence of elastin in collagen-based scaffolds has been shown to decrease scaffold stiffness[24] and modulate collagen contraction[25,26]. There is evidence suggesting that elastin can suppress the differentiation of proliferating fibroblasts into contractile myofibroblasts[27], thereby reducing wound contraction and modulating scar tissue formation.

Elastin does not adequately regenerate during severe wound healing and its distribution is disrupted in cutaneous scars[15]]. It takes 4-5 years for elastin expression to rise following cultured epithelial autograft (CEA) treatment of burn wounds. Elastin is functionally and spatially disorganized in scar tissue[28,29]. Expression of both elastin and fibrillin-1 are reduced in scar tissue with a particularly prominent reduction in hypertrophic scars[15]. Newly synthesized, elastic fibers in scar tissue always appear thin, fragmented, and less mature than elastic fibers in normal skin[15,29,30]. Even in scars older than 10 years, elastic fibers never reach the size and maturity of healthy skin[30], which attributes to the fact that hypertrophic scars are usually hard and inelastic[29]. The disruption of the elastic fiber system in healing wounds and scar tissue is well documented, but the mechanism behind this phenomenon is not clear. It is possible that elastin upregulation in healing wounds is not sufficient to regenerate robust elastin fibers. Elastin-containing dermal substitutes may improve the elasticity and functionality of severe scars by replacing the missing elastic network or by signalling the upregulation of elastic tissue biosynthesis. Consistent with this signalling role, dermal fibroblasts display increased elastin expression when they are stimulated with proteolytic digests of bovine elastin introduced into the skin of nude mice or into human skin explants[31].

Collagen-elastin composite scaffolds induce elastin deposition when implanted subcutaneously in rats, compared with collagen-only scaffolds that do not promote elastin synthesis[32,33]. The clinically best known human decellularized, collagen-elastin dermis is sodium chloride-sodium dodecyl sulfate-treated cadaver skin marketed as Alloderm®[34]. Alloderm® has been applied to human burns in a range of different procedures[34-37]. Alloderm®-grafted sites often show good cosmetic and functional results, with limited contractures observed on relatively small burn areas (<20% TBSA)[35]. Case studies also report increased skin elasticity and improved cosmetic appearance when Alloderm® is grafted with split thickness autografts,

compared with split-thickness autografts alone[34,35]. When applied to burned joints, Alloderm® can minimize wound contraction and allow joint movement[37]. Because of its high cost and limited quantity, Alloderm® is mostly used in reconstructive surgery to release skin contractures and hypertrophic scars[37]. The cost of Alloderm® as mentioned by Butterfield in a 2013 review article was 21.7 Euro/cm^2 [(38)].

Another dermal matrix consisting of native bovine collagen (type I, III and V) fibers was coated with 3% (w/w) a-elastin derived from bovine ligamentum nuchae, marketed as MatriDerm®. MatriDerm® in combination with a split-thickness mesh graft showed improved skin pliability and elasticity compared with split-thickness mesh grafts alone in scar reconstruction wounds. However, these benefits were not seen in burn wounds after 3 months[39]. In a scar follow-up study, no difference in scar elasticity was observed between MatriDerm®-grafted and control scars in the burn wounds at 12 years post grafting. However, there was a perceived improvement for MatriDerm® -grafted wounds compared with control wounds in subjective scar assessment conducted by patients and clinicians[38]. MatriDerm® has proved particularly useful in the treatment of hand burns, which are reported in 60-90% of burn cases[40,41]. A long-term follow-up of upper-extremity wounds treated with this scaffold in combination with a sheet autograft reveals good skin pliability, scar height, and ultimately, hand function[41]. Radu et al. found that MatriDerm® when used in combination with a split thickness autograft improved the range of motion and the quality of scars compared with split-thickness grafts alone[42]. The beneficial effects of MatriDerm®, including the reduction of wound contraction and stimulation of dermal regeneration, are believed to be conveyed in the early healing stages (within the first 2 weeks) through the inhibition of dermal fibroblast differentiation into contractile myofibroblasts[25].

MatriDerm® is a first step toward incorporation of soluble elastin derivatives in dermal substitute scaffolds. MatriDerm®, however, consists of a collagen scaffold coated with elastin, and its benefits are therefore not derived from the presence of an elastin fiber network or elasticity of the scaffold. The porous nature of the matrix may support a more rapid vascularization of the matrix, however the absence of elastin fibers and thus a network of elastin may also diminish its long-term beneficial effect in terms of elasticity. Further, the scaffold is composed of animal derived proteins, which carry risks of immune rejection and pathogen transfer as well as suffer from potential heterogeneity because of their batch-to-batch inconsistencies. The cost of MatriDerm® as mentioned by Lamy et al. in a 2013 article is on average 5.30 Euro/cm^2 [(43)].

Increasing understanding of the importance of elastin in tissue-engineered scaffolds has resulted in research into the elastin- and tropoelastin-based scaffolds. These scaffolds are currently undergoing *in vitro* and early *in vivo* testing[44]. In the clinical setting often logistic, financial and temporal issues continue to challenge the burn surgeon to use dermal substitutes on a more larger scale.

We set out to develop a dermal substitute from glycerol preserved allografts more than a decade ago, which was intended to have the following key advantages: native collagen and elastin matrix, easy storage and handling, inactivation of virus and

micro-organisms[45,46] and most importantly, a non-profit product that could be available to a larger number of patients. The extreme high cost of dermal substitutes today impedes their widespread application and benefit for those who need it the most. As clinicians in the field our chief aim was to develop a practical and affordable dermal substitute for burn, cancer and trauma victims.

The most favourable prototype Glyaderm® was tested in animal studies, which showed favourable results in a three stage procedure, allograft, Glyaderm®, autograft (manuscript in preparation). These promising results prompted the current pilot study and randomized comparison. There have been many reports attesting the benefits of various dermal substitutes. However, to our knowledge there has been no conclusive randomized trial which demonstrates a superior outcome of skin resurfacing with a dermal substitute and split skin graft over skin resurfacing with a skin graft alone. Most burn experts do not question the value of dermal substitution in surgical burn care and long-term results of patients attest the added value. Objective scar assessment and longer follow-up is elucidating this advantage, which is already clinically apparent. Our pilot study shows consistent, stable long-term results after 6 years with pliable skin after bi-layered skin restoration with Glyaderm®. Objective scar assessment showed a significantly improved elasticity of the skin in patients treated with Glyaderm® and skin graft compared to skin graft alone ($p = 0.003$).

Glyaderm® is the first cost-effective, non-profit, dermal references substitute that can be compared with currently available dermal equivalents. To our knowledge we are the first to show that laser Doppler imaging allows monitoring of vascular ingrowth in dermal substitutes such as Glyaderm®. Although most burn experts advocate the use of dermal substitutes, the challenge remains to objectively show the perceived benefit over split skin grafting alone. The evolving evaluation with objective scar assessment tools within these studies may help to further demonstrate this benefit in the near future.

A disadvantage in our initial studies with Glyaderm® was the necessity for three procedures to full wound closure. Direct application of Glyaderm® onto the wound bed without allograft wound bed preparation did not seem to be a viable option in either the animal studies nor the phase I pilot study as demonstrated by the 3 patients with a full thickness skin defect after radial forearm flap harvest where, following immediate application of Glyaderm®, we expected no problems in view of the healthy wound bed, but in the end there was no ingrowth of the dermal substitute. The animal studies had also pointed out that simultaneous application of Glyaderm® and autograft was not feasible. In Glyaderm® processing a relative dense elastin-collagen network is preserved. Budding capillaries need to penetrate this network before they can nourish the overlying autograft. In addition, the earlier Glyaderm® prototypes were relatively too thick and suffered from batch to batch inconsistencies inherent to variation in selection. Continuous research, monitoring of selection and development improved this process of graft selection and standardization.

A purpose designed laser tool is now used to ensure selection of dermis of uniform thickness. The laser accurately scans the distance between the optic and the table and the optic and the Glyaderm® subsequently placed upon the table, allowing the

difference in height to be the thickness. The optimal 0.2-0.4 mm thickness glycerol preserved dermis is now selected for processing into Glyaderm®.

Glyaderm® is currently applied with simultaneous skin grafting after wound bed preparation with allografts for 5 days. This improvement has a distinct favourable impact on morbidity and cost[47]. We have now modified the study protocol of a recent ongoing multicentre Glyaderm® study to allow for recruitment of patients with this shorter surgical procedure.

Glyaderm® is produced by the Euro Skin Bank, Beverwijk, The Netherlands, a non-profit tissue bank that also monitors Glyaderm® commercial distribution for burn care and reconstructive procedures. Euro Tissue Bank ensures the quality and non-profit distribution of the product backed by a clinical specialist advisory group to facilitate and promote clinical use.

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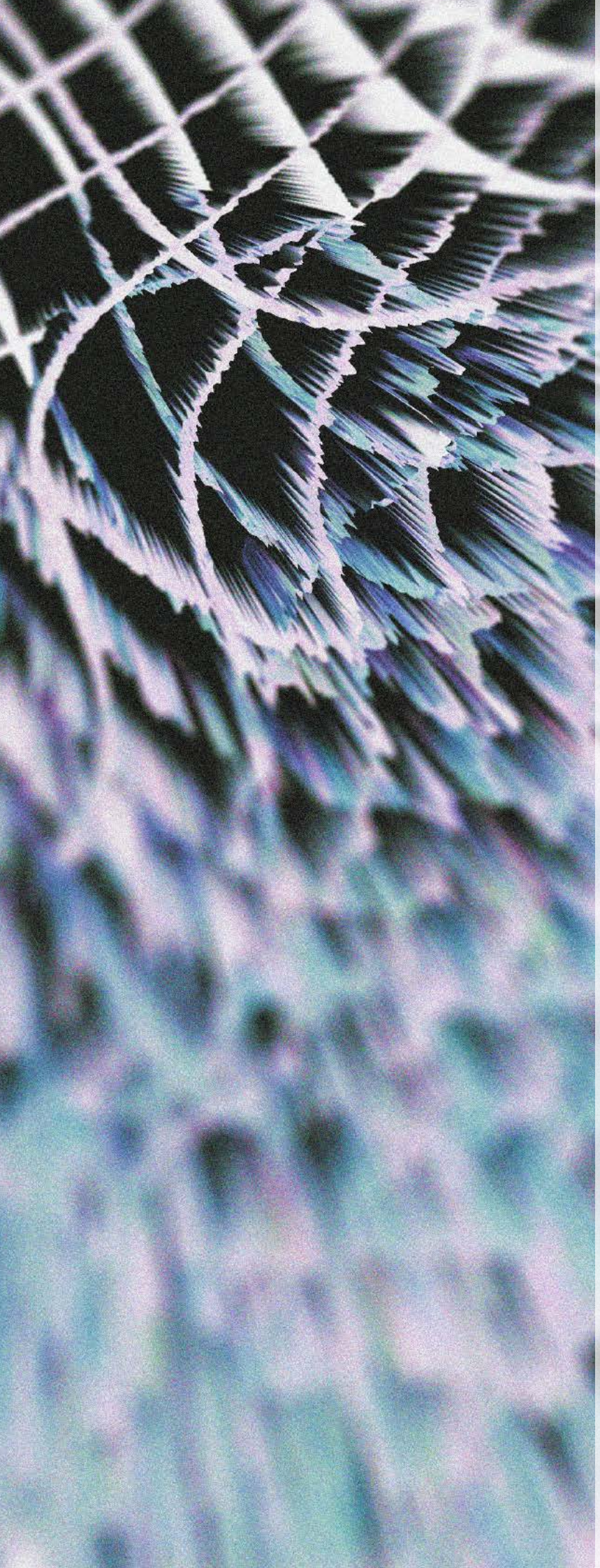

CHAPTER 9

VISUALISATION OF NEWLY SYNTHESISED COLLAGEN *IN VITRO* AND *IN VIVO*

NATURE. SCIENTIFIC REPORTS (2016)

C. OOSTENDORP
P.J.E. UIJTDEWILLIGEN
E.M. VERSTEEG
T.G. HAFMANS
E.H. VAN DEN BOGAARD
P.K.J.D. DE JONGE
A. PIRAYESH
J.W. VON DEN HOFF
E. REICHMANN
W.F. DAAMEN
T.H. VAN KUPPEVELT

Abstract

Identifying collagen produced de novo by cells in a background of purified collagenous biomaterials poses a major problem in for example the evaluation of tissue-engineered constructs and cell biological studies to tumor dissemination. We have developed a universal strategy to detect and localize newly deposited collagen based on its inherent association with dermatan sulfate. The method is applicable irrespective of host species and collagen source.

Introduction

Collagen, the most abundant protein family in the human body, plays a pivotal role in the organization of tissues and organs, and is a major determinant during organogenesis. In the field of tissue engineering and regenerative medicine, type I collagen is a key biomaterial[1] whereas in other fields, notably cancer research, collagen gels are frequently used in 3D studies to the migrational behavior of cells[2].

A common challenge in the field is to make a distinction between the collagen synthesized by cells and the (abundant) pre-existing collagen present in the biomaterial. Antibodies raised against collagens are of limited use due to the highly conserved nature of collagens[3] and the associated cross reactivity between collagen from different species. Other methods like metabolic radiolabeling and mass spectrometry[4] are laborious and do not provide information about the topography and organization of the newly synthesized collagen fibers.

In this study we evaluated newly synthesized fibrillar collagen (e.g. type I collagen), by making use of the inher- ent and intrinsic association of the glycosaminoglycan dermatan sulfate with collagen fibrils. Dermatan sulfate is the glycosaminoglycan part of the proteoglycans decorin and biglycan, which are both collagen fibril-associated molecules that play a role in the regulation of collagen fibril diameter. These proteoglycans remain present on the mature collagen fibril (**Figure 1a**, **cartoon**), and therefore dermatan sulfate is associated with collagen fibrils[5,6]. The technique described here is based on the selective detection of dermatan sulfate using the single chain variable fragment antibody GD3A12[7], combined with the absence of dermatan sulfate in experimentally or commercially produced biomaterials. We tested the technique both *in vivo* and *in vitro* using a number of collagenous biomate- rials including gels cultured with human fibroblasts with or without keratinocytes (denovoSkin® and denovoDerm® respectively)[8], experimental and commercially available scaffolds, and glycerol preserved acellular human dermis (Glyaderm®)[9].

Results

To evaluate the potential of the anti-dermatan sulfate antibody to identify collagen fibrils we applied immuno-electron microscopy using rat kidney cryosections. Antibody reactivity, as visualized by gold sphere-labeled protein A, was confined to collagen fibrils whereas other structures like cells and basement mem- branes did not stain (**Figure 1b**). Using immunofluorescence, antibody staining for dermatan sulfate was shown to co-localize with type I collagen, and was abolished by pretreatment of the sections with chondroitinase B, which specifically digests dermatan sulfate (**Figure 1c**).

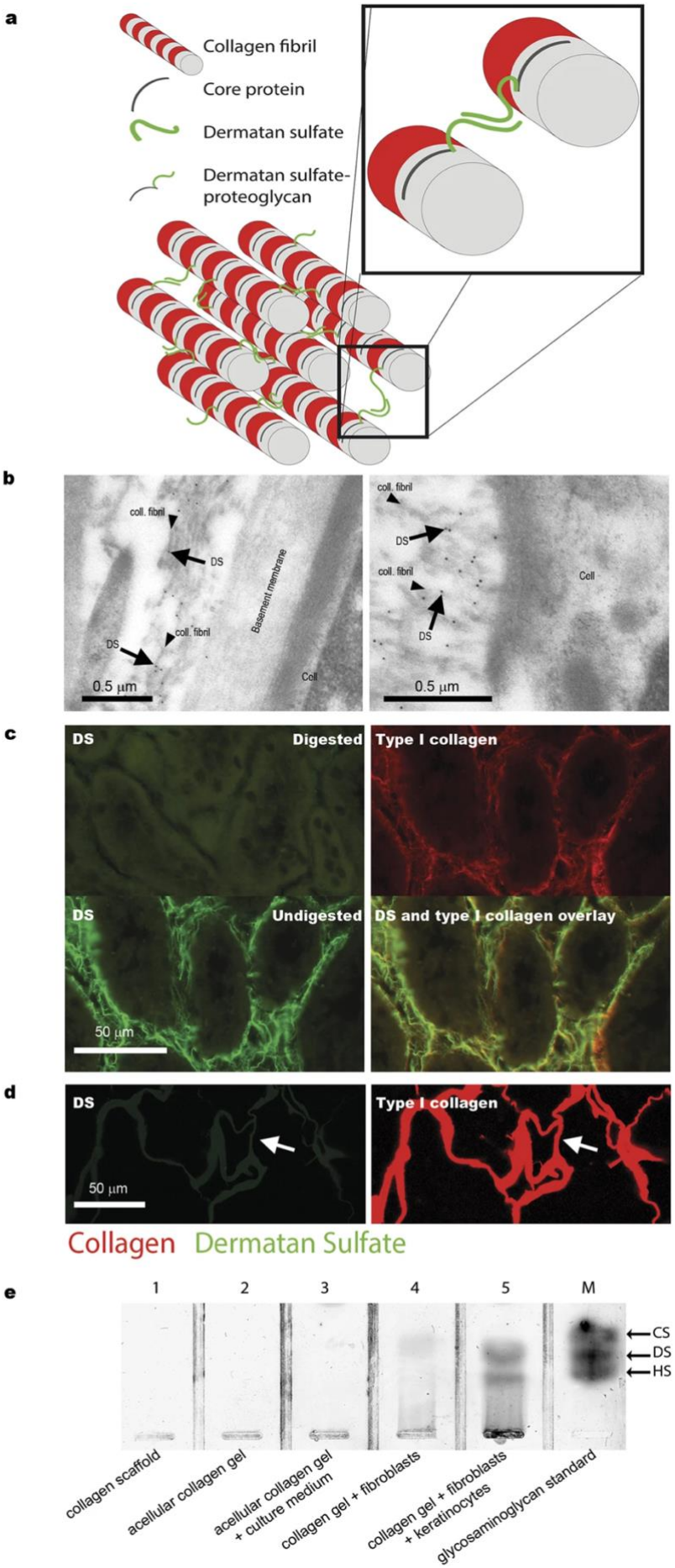

Figure 1. Overview and validation of strategy to identify newly synthesized collagen by dermatan sulfate. (a) Cartoon illustrating the intrinsic association of dermatan sulfate with collagen fibrils. (b) Identification of collagen fibrils using the anti-dermatan sulfate single chain antibody GD3A12. Arrows indicate immunogold labelling on collagen fibrils (rat kidney tissue, Bowman's capsule), but not on other structures such as cells and basement membranes. (c) Specificity of the anti-dermatan sulfate antibody as evidenced by loss of immunostaining after digestion of dermatan sulfate by chondroitinase B (rat kidney tissue). Note co-localization of dermatan sulfate and type I collagen. (d,e) Absence of dermatan sulfate in pre-seeded/ pre-implanted collagenous biomaterials as indicated by (d) immunostaining for dermatan sulfate (antibody GD3A12), (e) biochemical analysis of dermatan sulfate (agarose gel electrophoresis). In (d) arrows indicate identical areas stained for dermatan sulfate and type I collagen. In (e), lanes 1–3 represent acellular collagen gels/scaffolds, whereas lanes 4 and 5 represent cellularized gels. M, marker containing 5 ng each of chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS)®. coll.fibril: collagen fibril.

Absence of dermatan sulfate in the biomaterials. All collagenous biomaterials used were tested for the presence of dermatan sulfate, using immunohistochemical and/or biochemical techniques. Using immunofluores- cence, dermatan sulfate could not be detected in any of the biomaterials (**Figure 1d**, and supplementary **Figure S1**). In addition, using a highly sensitive silver staining method, dermatan sulfate could not be observed in collagen scaffolds (**Figure 1e**, lane 1) or in collagen gels (**Figure 1e**, lane 2 and 3).

Collagen deposition *in vitro* and *in vivo*. Having demonstrated the capacity of the antibody to detect collagen fibrils by virtue of its association with dermatan sulfate, and having established the absence of dermatan sulfate in collagenous biomaterials, we studied newly synthesized collagen fibrils produced by cells both *in vitro* and *in vivo*, using dermatan sulfate staining. Fibroblasts cultured *in vitro* in a collagenous gel produced collagen as evidenced by the presence of dermatan sulfate, which co-localized with type I collagen. Use of anti-type I collagen antibody did not discriminate between bovine collagen from the scaffold and the human collagen produced by the fibroblasts (**Figure 2a** 1–3). Dermatan sulfate staining, however, indicated the location of newly synthesized human col- lagen and was not present in the bovine scaffold collagen. Dermatan sulfate was also identified biochemically, and was detected only in cellularized collagen gels, and not in gels without cells (**Figure 1e**, lane 4). The location of newly synthesized collagen was time dependent, and initially present only at the perimeter of the fibroblasts (**Figure 2b** 1). At later stages (e.g. 12 days of culturing) collagen was also located further away from the cells, and eventually most of the original gel contained newly synthesized collagen (**Figure 2b** 4). These results were confirmed biochemically, showing increased amounts of dermatan sulfate as a function of time (**Figure 1e**).

Glycosaminoglycans are evolutionary highly conserved structures that are found throughout vertebrates as well as invertebrates[10]. It may therefore be expected that the anti-dermatan sulfate antibody can be used irrespective of the species that deposits the collagen. To evaluate this we stained collagen scaffolds implanted in different animal species and in humans. The following samples were used:

1. A flat collagen scaffold implanted subcutaneously in mice.
2. A tubular collagen scaffold implanted in the ureter of pigs.
3. A commercial collagen-chondroitin sulfate skin substitute (Integra®) implanted in a full-thickness skin defect in rats[11].
4. Integra® implanted in a soft tissue palatal defect in dogs[12].
5. Glyaderm® (acellular human dermis) implanted in a full-thickness skin defect in mice[13].
6. Glyaderm® clinically applied in full-thickness skin defects in humans[14].

We were able to visualize the dermatan sulfate (and hence newly deposited collagen) in all species tested, indicating the robustness and species independency of the procedure (**Figure 2** c,d). In line with the ingrowth of cells from the surrounding tissue into the scaffold, newly deposited collagen fibers were most prominent at the border of the scaffold, whereas deeper in the scaffold they were thinner and less

abundant (**Figure 2** c1). Small collagen deposits could easily be identified (**Figure 2** c2). The newly formed collagen was generally oriented in the same direction as the fibers from the original scaffold (e.g. in a parallel orientation, see **Figure 2** c3). In the pig model (**Figure 2** d1), newly formed collagen was clearly present alongside the collagen fibers of the original implanted tubular collagen scaffold. Newly formed collagen fibers could also be easily identified in rats and dogs after implantation (7 and 28 days respectively) with Integra® (**Figure 2** d2,d3). Integra® itself was not stained by the anti-dermatan sulfate antibody (supplementary Figure S1), even though the closely related glycosaminoglycan chondroitin sulfate is abundantly present in this commercially available skin substitute. The method was also applicable using the human skin derived Glyaderm®. Full-thickness wounds in mice treated with Glyaderm® [13] showed deposition of new collagen 8 days after implantation (**Figure 2** c4). Please note that due to the strong autofluorescence of elastic fibers in Glyaderm® we used bright field instead of fluorescence microscopy. Finally, the method was probed in a clinical setting in which burn patients were treated using Glyaderm®[14]. Biopsies taken 7 days after implantation clearly show new collagen in the dense collagenous environment of Glyaderm® at the border of the wound bed (**Figure 2** d4). Collagen fibers of the surrounding native tissue (i.e. in tissue not formed within the biomaterial) were also positive for dermatan sulfate in all species tested, as expected.

Discussion

The results presented above indicate that the anti-dermatan sulfate antibody GD3A12 is suitable to species inde- pendently detect newly formed collagen. Previously, analysis has been hampered by the inability to (immuno) histologically distinguish newly formed collagen from biomaterial/scaffold collagen. The technique described here offers a solution to this problem. However, it is not without potential pitfalls. Although the vast majority of dermatan sulfate is associated with collagen as part of the proteoglycans decorin and biglycan[5], a small fraction may be present associated with other structures such as elastin and fibrillin-containing microfibrils[15]. In addition, dermatan sulfate may be part of the proteoglycan versican[16] associated with elastic fibers[17]. However, in this study we did not observe any association of dermatan sulfate with elastic fibers detected either by autofluorescence or by anti-elastin antibodies (see double staining with dermatan sulfate, supplementary **Figure S2**), indicating that such an association was not present in the tissues studied here.

Next to the obvious use in regenerative medicine, the proposed method may be applied to other fields of research including cancer biology. Tumor cells spread and invade into the surrounding tissues while remodeling the extracellular matrix. It has been suggested that in doing so tumor cells make use of newly deposited collagen fibrils[18]. A widely used 3D model to study the migrational behavior of tumor cells is the use of collagen gels. The technique described here may be of value in further defining the role of newly formed collagen fibrils in tumor biology using such models[2].

In conclusion, the detection of newly synthesized collagen based on its association with dermatan sulfate and applying the single chain antibody GD3A12 represents an inexpensive, fast and easy technique to evaluate the presence and orientation of de novo synthesized collagen fibrils in collagen based biomaterials. As such it can be applied in many research areas including tissue engineering and tumor biology.

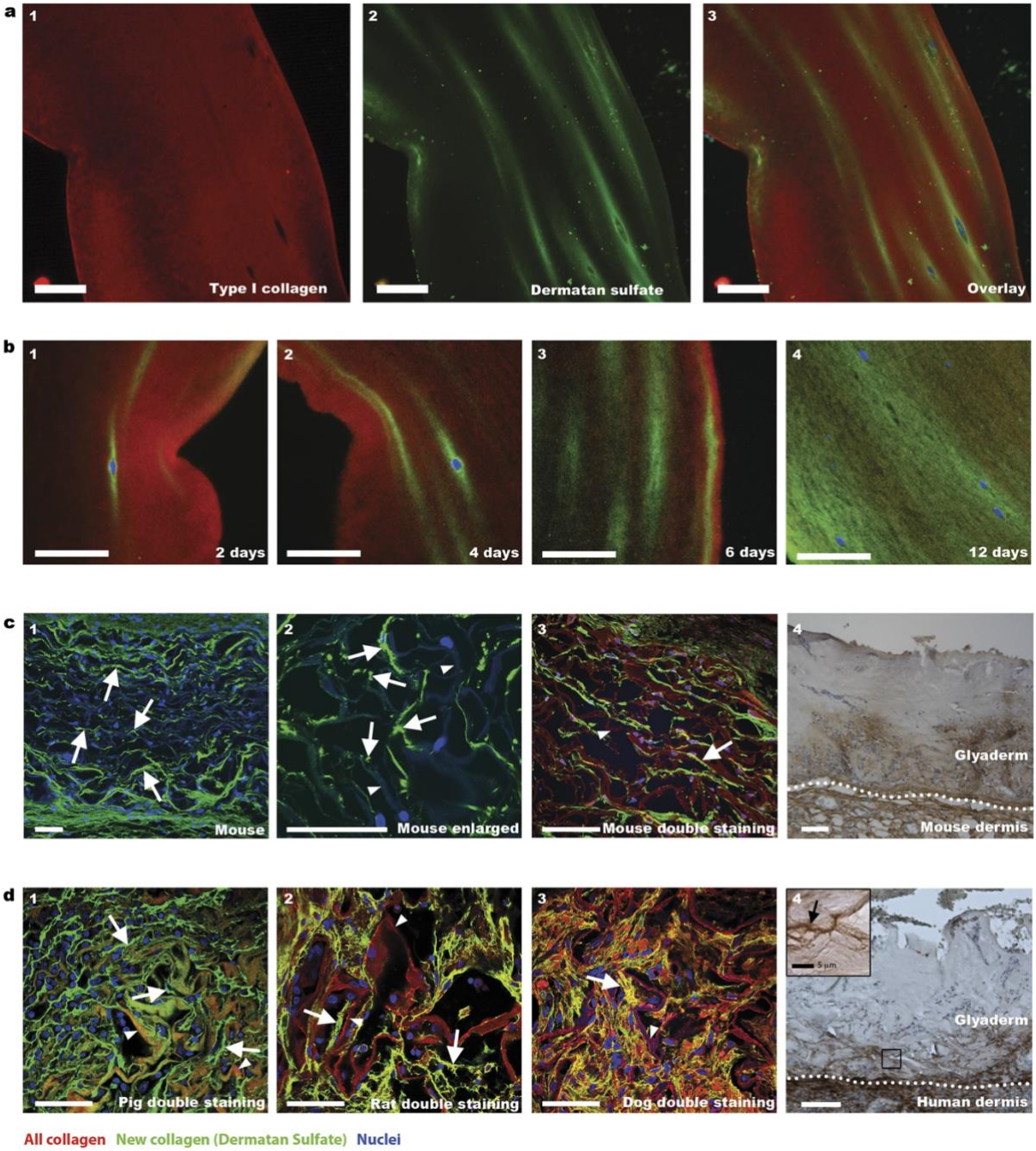

Figure 2. Detection of newly synthesized collagen fibrils in cellularized/implanted collagenous biomaterials. (a) Collagen gel cultured for 6 days with human fibroblasts. Newly deposited collagen is indicated by green dermatan sulfate staining (a2,a3), whereas all collagen is indicated by red type I collagen staining (a1,a3). (b) Location of newly deposited collagen in collagen gels cultured in time with fibroblasts/keratinocytes. Note increase of new collagen over time (b1-4). (c) Newly deposited collagen fibrils (arrows) in a collagen scaffold (arrowhead), two weeks after subcutaneous implantation in mice (c1-3) (for clarity, autofluorescence of background collagen was enhanced). For Glyaderm®

(acellular human dermis), new collagen is indicated by brown staining (c4). (d) Newly deposited collagen fibrils (arrow) in various species (d1-4) after implantation of a collagen scaffold (arrowhead) in pig (1 month) (d1), Integra® (arrow head) in rat (1 week) (d2), Integra® (arrow head) in dog (4 weeks) (d3), and Glyaderm® in human (inset shows fibrillar structure) (d4). Scale bars are 50 μm unless indicated otherwise.

Materials and methods

Materials

Papain, barium acetate, paraformaldehyde, 3,3′-diaminobenzidine tetrahydrochloride (DAB), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and protein A were from Sigma, St. Louis MO, USA. Chondroitinase B was from IBEX, Montreal, Quebec, Canada. Agarose and gel bond film were from Lonza, Rockland, USA, and Tissue Tek from Sakura Finetek Europe BV, Alphen aan den Rijn, The Netherlands. Tris-HCl was from Invitrogen, Carlsbad, CA, USA. Sodium chloride and magnesium acetate were from Merck, Darmstadt, Germany. Lowicryl HM20 was from Aurion, Wageningen, The Netherlands and Mowiol 4-88 mounting medium was from Calbiochem, San Diego, CA, USA. Bovine serum albumin (BSA) was from PAA laboratories, Pasching, Austria. The following antibodies were used: rabbit anti-bovine type I collagen IgG from Millipore, Cambridge, UK; mouse anti-bovine elastin IgG from Sigma; rabbit anti-VSV IgG from Rockland, Gilbertsville, PA, USA; mouse anti-VSV IgG from mouse hybridoma cell line P5D4 from the American Type Culture Collection, Rockville, MD, USA; peroxidase labeled mouse anti-VSV IgG from Sigma and mouse anti-Penta-His IgG from QIAGEN GmbH, Hilden, Germany; goat anti-mouse IgG Alexa Fluor 488 conjugated and goat anti-rabbit IgG Alexa Fluor 594 conjugated from Invitrogen, Eugene, OR, USA. The single chain variable fragment antibody GD3A12 selective for dermatan sulfate was obtained as described[7,19]. As a source of this antibody, periplasmic fractions isolated from bacteria expressing the antibody were used[20]. The antibody contains a VSV and a HIS tag.

Histology

Paraffin-embedded and frozen tissues were sectioned at 5μm thickness. Paraffin sections were deparaffinized in xylene for 3 × 5 min, followed by a descending series of ethanol and processed for immunohis- tochemistry. Cryosections were air-dried before staining.

Immunofluorescence staining

To stain for dermatan sulfate, deparaffinized sections were blocked for 15 min with 1% BSA in Tris buffered saline (TBS, 50 mM Tris-HCl pH 7.0 containing 150 mM NaCl). All further incubations were performed at ambient temperatures for 45 min and sections were washed 3 × 5 min with TBS in between incubations. Antibodies

were diluted in 1% BSA in TBS. Paraffin sections were incubated with antibody GD3A12 (1:5 - 1:20), followed by incubation with mouse anti-VSV antibody P5D4 (1:10) and an Alexa Fluor 488-conjugated goat anti mouse antibody. For double staining with type I collagen, antigen retrieval using citrate buffer was applied. Paraffin sections were pretreated with citrate (10 mM sodium citrate, pH 6.0) for 20 min at either 95 °C (tissues) or ambient temperature (cultured gels). In one occasion boiling temperature was used, but this caused damage to the sections. Sections were extensively washed with TBS to remove the citric acid buffer and blocked with TBS/BSA. Anti-type I collagen antibody (1:500–1:1500) was applied and visualized using an Alexa Fluor 594-conjugated goat anti-rabbit antibody. For detection of dermatan sulfate in implanted collagen scaffolds in mice, a rabbit anti-VSV antibody (1:500) was used and visualized using goat anti-rabbit IgG Alexa Fluor 488. In the case of double staining of dermatan sulfate and type I collagen in mice, dermatan sulfate was visualized using Alexa Fluor 488 conjugated mouse anti-Penta-His antibody.

For double staining of sheep skin cryosections for elastin and dermatan sulfate, the sections were air-dried for 30 min. Hereafter, the procedure as described above was applied. Mouse anti-bovine elastin antibody was diluted 1:200 and visualized using goat anti-mouse IgG Alexa Fluor 594. Dermatan sulfate was visualized using rabbit anti-VSV IgG and goat anti-rabbit IgG Alexa Fluor 488.

For visualization of the nuclei, sections were incubated for 15 min with DAPI (10 µg/ml in PBS). After extensive washings with PBS, the sections were enclosed with Mowiol mounting medium.

For detection of dermatan sulfate in the human skin substitute Glyaderm®, containing autofluorescent elastic fibers, peroxidase conjugated mouse anti-VSV IgG (1:100) and DAB were used. Omission of the antibody GD3A12 was taken as a control, and was negative in all cases.

Digestion of dermatan sulfate to evaluate specificity of GD3A12

To evaluate the specificity of the antibody GD3A12 for dermatan sulfate, cryosections were digested overnight at 37 °C with 20 mU/ml chondroitinase B in 25 mM Tris-HCl pH 8.0 containing 2 mM magnesium acetate. The next day, the digestion was repeated with 20 mU/ml chondroitinase B for another 2 h. As a control, sections were incubated in buffer without enzyme.

Microscopic imaging, equipment and settings

Images of the *in vitro* cultured collagen gels were taken with a Leica DM6000 B microscope equipped with a Leica DFC 480 camera (20x objective), using Leica Application suite V4.3.0. The exposure time was kept constant for all measurements. DAPI, Alexa Fluor 488 and 594 were excited with a mercury HBO100 lamp using the excitation filters BP410/15 nm BP490/20 nm and BP562/40 nm respectively, and

the emission was collected after filtering with 430, 500, 593 dichroic mirrors respectively.

All other images were captured with the Olympus FV1000 Confocal Laser Scanning Microscope. Photos were imaged using a 20× objective and a 60× objective. DAPI, Alexa Fluor 488 and Alexa Fluor 594 were excited at 405 nm, 488 nm and 559 nm, respectively. Using a combination of the beam splitters SMD490 and SDM560, the emission was collected with the emission filters BA430-470, BA505-540 and BA575-675 for DAPI, Alexa Fluor 488 and Alexa Fluor 594, respectively.

For **Figures 1c and 2a,b** and supplementary **Figure S1a**, the microscope settings were as follows: space resolution 2560 × 1920, pixel dimension 0.012 pixels/μm, image depth 32 (RGB), excitation filters: 410/15 (DAPI), 490/20 (AF488), 562/20 (AF594) and gamma correction was set at 1. For **Figures 1d and 2c,d**, the settings were as follows: space resolution 1024 × 1024, pixel dimension 1.61 pixels/μm, image depth 32 (RGB), excitation filters 405 (DAPI), 488 (AF488), 559 (AF594), emission filters BA430-470 (DAPI), BA505-540 (AF488), BA575-675 (AF594). Dichroic beam splitters 490 9DAPI) and 560 (AF488) were used. No gamma correction was applied.

Image processing was performed using ImageJ 1.48v (National Institutes of Health, USA). Before merging, both brightness and contrast were adjusted similarly for all photos including the controls.

Immuno-electron microscopy

To evaluate the reactivity of the antibody for dermatan sulfate on collagen fibrils, immuno-electron microscopy was performed on lowicryl HM20 embedded rat kidney samples[21]. The tissue was incubated for 3 h in Somogyi solution [0.1 M phosphate buffer (pH 7.3) containing 4% formaldehyde, 0.05% glutaraldehyde and 0.2% picric acid][22]. After cutting, 200 μm sections were frozen in liquid propane at −190 °C.

Using freeze-substitution (Leica-KF80) the sections were embedded in lowicryl HM20. Ultrathin sections were mounted on nickel grids. For immunostaining sections were blocked with 0.25% BSA in phosphate buffered saline (BSA/PBS), followed by an overnight incubation at 4 °C with antibody GD3A12 (5× diluted periplasmic fraction in BSA/PBS). After washing, bound GD3A12 was visualized using 10 nm gold-sphere labeled protein A (1:400 in BSA/PBS) prepared according to Slot et al.[23]. Subsequently, sections were washed in PBS, post-fixed for 5 min in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), washed with distilled water, and post-stained with uranyl acetate. Sections were examined using a JEOL 1010 electron microscope.

Agarose gel electrophoresis

To analyze the presence of glycosaminoglycans including dermatan sulfate in collagen scaffolds/gels, agarose gel electrophoresis was performed. To 40 mg dry weight of the samples, 2.5 U/ ml papain was added to a total volume of 500 μl in

order to digest proteins. 0.5 µl of the samples was loaded on a 1 mm thick 1% agarose gel in 50 mM Ba(Ac)2, pH 5.0, casted on a gel bond film. A marker was included containing 5 ng of chondroitin sulfate (CS), 5 ng dermatan sulfate (DS) and 5 ng heparan sulfate. The gel was run at 30 mA in electrophoresis buffer (50 mM Ba(Ac)2, pH 5.0) until the front of the loading dye had moved about 8 cm into the agarose gel. Subsequently, the agarose gel was stained with silver as described by Van de Lest et al.[24].

Gels and scaffolds

All experimental protocols described by Sun et al.[25], Nillesen et al.[11], van Kilsdonk et al.[13], de Jonge et al.[26], and Ophof et al.[12] were approved by the Institutional Animal Welfare Committee (DEC) of the Radboud university medical center, Nijmegen, The Netherlands. The experimental procedures described by Braziulis et al.[8] were approved by the Ethics Committee of the Canton Zürich (KEK), Switzerland. The study protocol as described by Pirayesh et al.[14] was approved by the Ghent University Hospital Ethics Committee.

All experiments were carried out in accordance with the guidelines of the Institute of Laboratory Animal Research[27] and the declaration of Helsinki principles.

Cellularized collagen gels

The dermal (denovoDerm) and dermal-epidermal (denovoSkin) collagen gels were prepared using bovine type I telocollagen seeded either with primary fibroblasts only (denovoDerm), or with a combination of primary fibroblasts and keratinocytes (denovoSkin), isolated from skin biopsies from 3 individuals who had given informed consent, as described by Braziulis et al.[8]. 50,000 fibroblasts were cultured in DMEM sup- plemented with 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES), 90 µg/ml streptomycin and 90 U/ml penicillin (all compounds from Invitrogen, Basel, Switzerland) for 2, 4 or 6 days in compressed collagen gels at 37 °C and 5% CO2. For the dermal-epidermal skin substitute, 500,000 keratinocytes were seeded on top of the dermal substitute and cultured for another 6 days (12 days in total) in serum free keratinocyte medium. Samples were fixed in 4% paraformaldehyde and embedded in paraffin.

Acellular collagen scaffolds implanted in mice. Flat porous collagen scaffolds were prepared as described by Sun et al.[25] from a 0.4% (w/v) type I collagen suspension in 0.25 M acetic acid. After homogenization, the suspension was pipetted in a polystyrene mold, frozen and lyophilized resulting in porous collagen scaffolds. Hereafter, the collagen scaffolds were pre-incubated in 50 mM 2-(N-morpholino)ethanesulphonic acid pH 5.0 (MES) and crosslinked using 33 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 6 mM N-hydroxysuccinimide (NHS) in 50 mM MES buffer containing 40% ethanol for 4 h. After multiple washing steps with subsequently 0.1 M Na2HPO4, 1 M NaCl, 2 M NaCl, and water, the scaffolds were frozen at −20 °C and lyophilized, followed by sterilization by γ-irradiation (25 kGy, Synergy Health, the Netherlands). Collagen

scaffolds were incubated in sterile 0.9% NaCl and subcutaneously implanted in 7-weeks-old Balb/cByj mice. After two weeks, mice were sacrificed and tissue was dissected around the site of implantation, fixed in 4% paraformaldehyde and embedded in paraffin.

Tubular acellular collagen scaffolds implanted in pigs. Tubular collagen constructs of 6 cm in length and an inner diameter of 6 mm were prepared as described using a 0.5% (w/v) collagen suspension in 0.25 M acetic acid[28]. After lyophilization the tubes were crosslinked followed by extensive washing, as described above. Tubular scaffolds were kept in 70% ethanol before γ-sterilization. Sterilized tubular collagen scaffolds were implanted in the ureter of 4-months-old pigs, dissected after one month, fixed in 4% paraformaldehyde and embedded in paraffin[26].

Integra® implanted in rat and dog. In rats, Integra®, a commercially available collagen-chondroitin sulfate scaffold[29], was implanted in a full thickness wound[11]. After 7 days, rats were sacrificed, the wound area dissected, fixed in 4% paraformaldehyde and embedded in paraffin[11]. In dogs, Integra® was implanted according to the Von Langenbeck procedure for palatal repair[12]. Samples were taken 28 days post implantation. Tissue was fixed in 4% formaldehyde, decalcified in 20% formic acid and 5% sodium citrate[12] and imbedded in paraffin.

Glyaderm® implanted in mouse and human. Glyaderm®, a glycerol preserved acellular human dermis containing native collagen and elastic fibers[9] was produced by the Euro Skin Bank, Beverwijk, The Netherlands. Full thickness wounds at the back of 8-week-old mice were implanted with Glyaderm®. After 8 days, mice were sacrificed,fixed in 4% paraformaldehyde and embedded in paraffin[13].

Application of Glyaderm® to a human wound bed was performed and described by Pirayesh et al.[14]. Informed consent was given by all patients. Full-thickness defects were engrafted with Glyaderm®. After 1 week, biopsies were fixed in 4% paraformaldehyde and embedded in paraffin.

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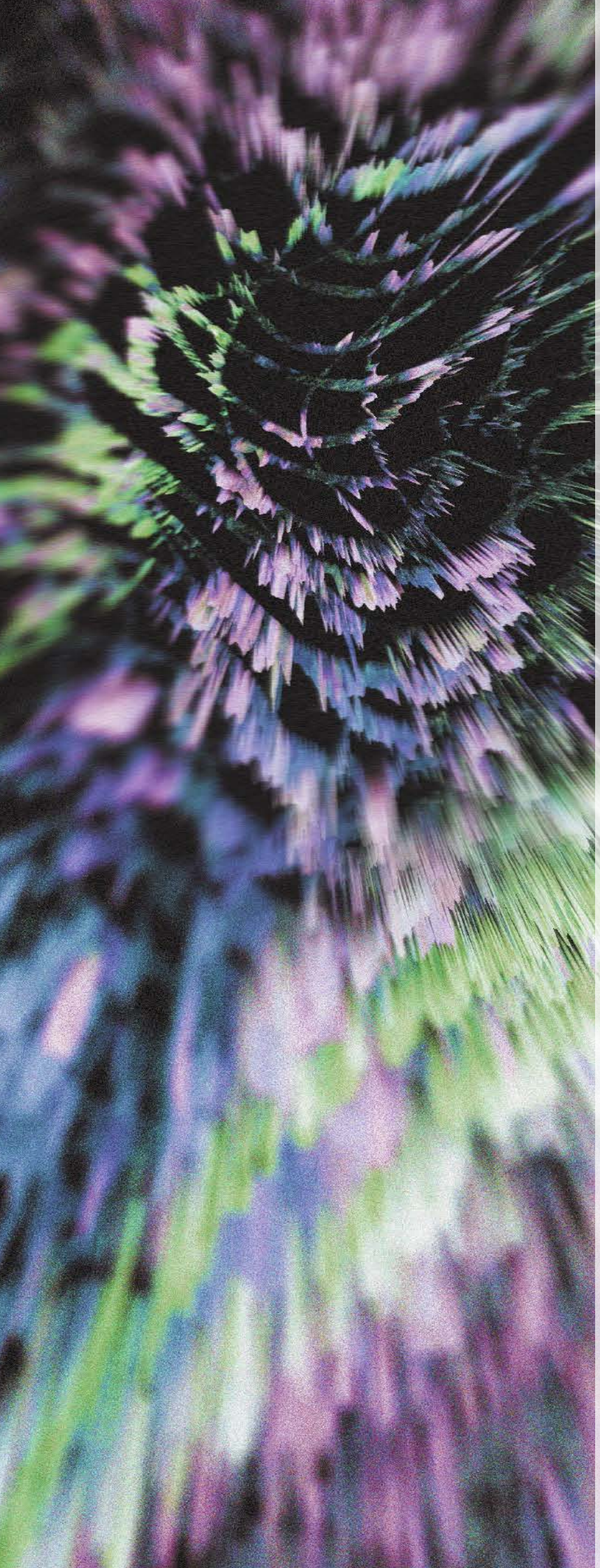

CHAPTER 10

A SINGLE STAGE BILAYERED SKIN RECONSTRUCTION USING GLYADERM AS A DERMAL REGENERATION TEMPLATE, RESULTS IN IMPROVED PATIENT SATISFACTION

BURNS & TRAUMA (2023)

I. DE DECKER
H. HOEKSEMA
J. VERBELEN
P. DE CONINCK
M. SPEECKAERT
S. DE SCHEPPER
P. BLONDEEL
A. PIRAYESH
S. MONSTREY
K.E.Y. CLAES

Abstract

Background

Absence of almost the entire reticular dermal layer is inherent to the use of autologous split-thickness skin grafting (STSG) to close full thickness wounds, often resulting in hypertrophic scars and contractures. Many dermal substitutes have been developed, but unfortunately most have varying results in terms of cosmetic and/or functional improvement as well as overall patient satisfaction, in addition to prohibitively high costs in the majority of countries worldwide. Bilayered skin reconstruction using the human-derived glycerolized acellular dermis (Glyaderm®) has already been reported to result in significantly improved scar quality using a two-step procedure. Unlike the necessary two-step procedure for most commercially available dermal substitutes, in the current study we aimed to investigate the use of Glyaderm® in a more cost-effective single-stage engrafting. A method which, if autografts are available, is preferred by the majority of surgeons.

Methods

A prospective, randomized, controlled, intra-individual, single-blinded study was performed, investigating the simultaneous application of Glyaderm® and STSG versus STSG alone in full-thickness burns or comparable deep skin defects. During the acute phase bacterial load, graft take, and time to wound closure were assessed and were the primary outcomes. Aesthetic and functional results (secondary outcome) were evaluated at 3, 6, 9, and 12 months follow-up using subjective and objective scar measurement tools. Biopsies for histological analysis were taken at 3 and 12 months follow-up. Results: Sixty-six patients representing 82 wound comparisons were included. Graft take (>95%), pain management, and healing time were comparable in both groups. At 1 year follow-up, the overall POSAS assessed by the patient, was significantly in favour of sites where Glyaderm® was used. Not infrequently, patients attributed this difference to improved skin sensation. Histological analysis showed the presence of a well-formed neodermis, with donor elastin present up to 12 months.

Conclusion

A single-stage bilayered reconstruction with Glyaderm® and STSG results in optimal graft take without loss of Glyaderm® or the overlaying autografts due to infection. In addition, the presence of elastin in the neodermis was demonstrated during long-term follow-up in all but one patient, which is a crucial factor contributing to the significantly improved overall patient satisfaction.

Background

The established treatment of deep partial and full-thickness burns consists of early removal of non-viable tissue followed by skin grafting[1–3]. This approach resulted in a mortality reduction in major burns and is essential in modulating the body's physiologic response, reducing the risk of bacterial colonization and infection and shortening the length of hospital stay[1,4,5]. Inherent to the use of split- thickness skin grafts (STSG) to close these deep defects, is the almost complete absence of the deeper dermal layer which often leads to hypertrophic scar formation (HTS) with reported incidences ranging from 32 to 72 percent post-burn[6–15]. The restoration of normal skin function and cosmesis is the holy grail for every burn surgeon and an important step in achieving this goal is the use of dermal substitutes[16]. Dermal substitutes or Dermal Regeneration Templates (DRTs) aim to improve dermal restoration by providing a neo-dermis that anatomically functions more like natural dermis rather than fibrotic tissue, therefore, improving scar characteristics and increasing the patients' quality of life (QoL)[5,16]. A wide variety of synthetic and biological dermal substitutes are currently available and they are classified according to scaffold type, thickness, number of layers, cell types, period of application and the type of wound to be treated[17]. A DRT plays the simultaneous role of a supporting structure and an extracellular matrix (ECM) by providing a scaffold for the formation of a permanently integrated neo-dermis[4,5,17]. Ideally, dermal templates allow effective fibroblast migration, adequate endothelial cellular influx for the creation of a vascular network, cell proliferation, secretion of native collagen, and the timely degradation and proper formation of new tissue architecture[4,5,17]. The neodermis that creates the framework of the wound needs to be flexible, elastic, able to withstand shear forces, and must ensure wound stability for a considerable amount of time[17]. From a surgeon's perspective, a DRT provides immediate wound coverage post-excision, establishes a barrier preventing fluid loss and allows the use of an ultra-thin autograft reducing donor site morbidity[5].

Many of the commercially available DRT's focus on supplying a three-dimensional (3D) fiber network primarily based on collagen from either xenogenic, allogenic, or synthetic origin[18,19]. At the same time, these DRTs are restricted by lack of elasticity and impaired by scaffold contraction[18]. Surprisingly, elastin historically has been inadequately represented in commercial dermal substitutes even though it plays an indispensable role in skin structure and function, mainly determining its resilience, texture, and quality[18]. Elastin has inherent cell signalling properties, promoting responses including chemotaxis, cell attachment, proliferation, differentiation and has the potential to limit cellular contractile forces[18,20,21]. Although dermal fibroblasts are inherently capable of secreting the protein monomer elastin, its synthesis is repressed by post-transcriptional mechanisms[22,23]. Moreover, the dermal elastin network does not regenerate adequately after severe wound healing and even in scars older than a decade, newly synthesized elastin fibers remain fragmented and never reach mature size, correlating with the hard and inelastic nature of HTS[18,24]. Increasing cicatrix quality and especially improving scar elasticity through dermal replacement in the

reconstruction of full-thickness skin defects should therefore incorporate a well-preserved 3D collagen-elastin fiber network[25]. A few collagen-elastin DRTs of human or allogeneic origin are commercially available e.g. Alloderm®, Dermamatrix®, Surederm® and Glycerolised Acellular Dermis (Glyaderm®)[26,27]. Glyaderm® is preserved in a glycerol solution which has been shown not to harm the skins structures and has virucidal properties when incubated and viral particle survival rates are extremely low[24,28–30]. Irradiation is a different technique of sterilization that only has a minor impact on the antigenicity of the skin and moreover it stiffens and damages the skin by inducing collagen cross-links impeding the skin from properly adhering to the wound bed due to the creation of free radicals[28,31]. For the storage of tissue, there is also the option of freezing the skin with liquid phase nitrogen, called cryopreservation. However processing skin with glycerol is simpler, more cost-effective and additionally has antimicrobial and antiviral properties[30]. Due to the low-cost incubation and preservation methods, Glyaderm® offers a cost-effective method for dermal substitution in deep partial and full-thickness skin defects. Improvement of scar quality using Glyaderm® as a DRT in a two-step procedure has been demonstrated in a phase III clinical trial including patients[24]. In the study described here, we investigated the use of Glyaderm® in a single-stage setting for the bilayered skin reconstruction of deep or full-thickness burns and comparable skin defects (**Figure 1**).

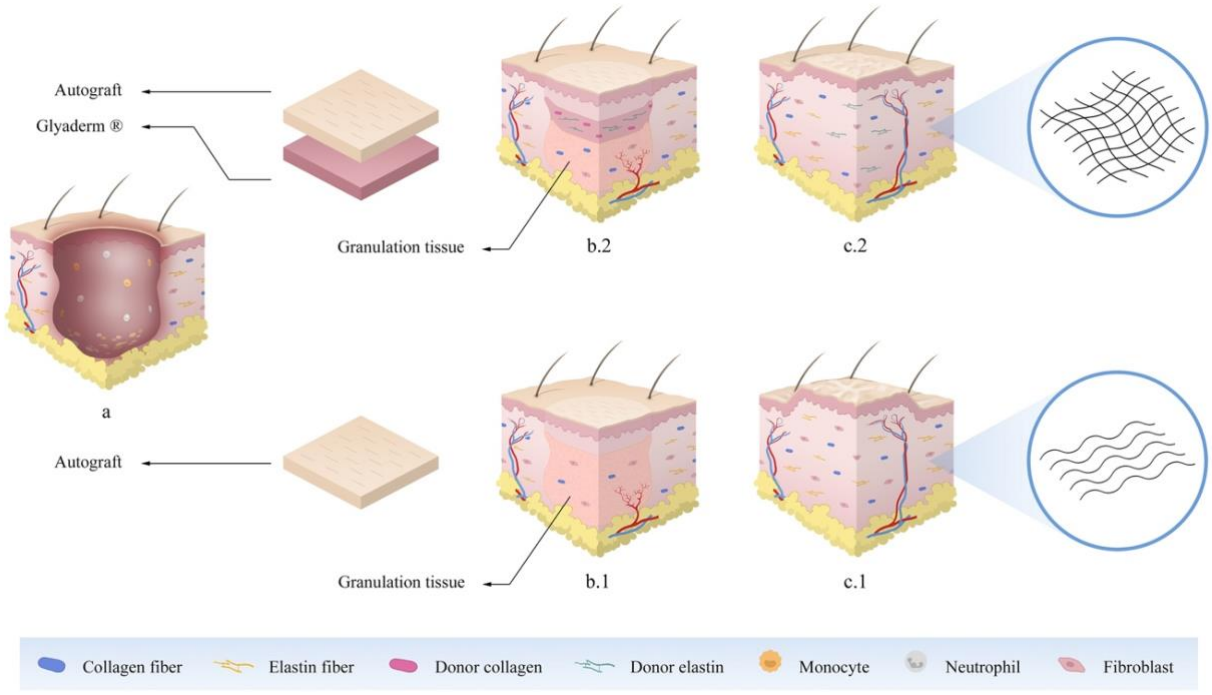

Figure 1. Artistic illustration of the immediate simultaneous bilayered skin reconstruction using Glyaderm® as a dermal substitute on a full-thickness skin defect. Defects are closed after proper wound bed preparation. (a) Defect with epidermal and dermal component involved (b.1) Single layer reconstruction by autografting without placement of a dermal substitute. (b.2) Simultaneous bilayered reconstruction using Glyaderm® and autografts. Subsequent vascularization of the dermal substitute. (c.1) Spatial orientation of the fibers is

crude and parallel. Scar shows more contracture and hypertrophy compared to the bilayered skin reconstruction. (c.2) Spatial orientation of the fibers is similar to the natural basket-weave pattern due to Glyaderm® acting as a guide for infiltrating cells. Due to this, the scar shows less contracture and hypertrophy compared to autograft alone.

Methodology

Ethics committee

This study was approved by the local ethics committee (B670201733327) and eligible patients were included after obtaining informed consent. Glyaderm® was produced and supplied by the Euro Skin Bank (Beverwijk, The Netherlands). The production process of Glyaderm® has been published by Richters et al.[24].

Study design

This study was a randomized, controlled, single-blind, intra-individual comparison of deep dermal and full-thickness skin defects engrafted simultaneously with Glyaderm® and STSG (intervention) versus STSG alone (conventional treatment) in a monocentric setting.

The primary study outcome measures were the evaluation of autologous graft take on day 5-7 post- operative comparing Glyaderm® and STSG versus STSG alone, the comparison of healing time between the two procedures and the assessment of the bacterial load. Secondary outcome measures were the functional and aesthetic outcome of a single-stage bilayered skin reconstruction using Glyaderm® and STSG versus STSG alone. Secondary outcome measures were evaluated with objective and subjective tools at 3, 6, 9, and 12 months follow-up after achieving wound closure.

Patient recruitment

107 Patients for this clinical trial were included from the period of February 2017 up until August 2020. The last follow-up took place in September 2021. A detailed overview of the eligibility criteria can be found in **Table 1**.

Inclusion criteria
• All deep partial thickness and full thickness burns as shown by laser Doppler imaging (LDI) and/or clinically evaluated by two plastic surgeons or burn care coordinator • Other full-thickness skin defects besides burns e.g. necrotizing fasciitis, deglovements or phalloplasty donor sites after free flap harvest • Possibility to follow the complete treatment schedule until final graft take and subsequently wound healing and participation in the follow-up schedule • Informed consent has been obtained • Age between 18-80 years
Exclusion criteria
• All partial thickness burns that can heal by conservative treatment confirmed by LDI • Not completing the treatment schedule or declining further follow up • The patient has any condition(s) that seriously compromises the patient's ability to complete this study • The patient has participated in another study utilizing an investigational drug within the previous 30 days • The patient has one or more medical condition(s), diabetes, including renal, hepatic, hematologic, neurologic, or immune disease that in the opinion of the investigator would make the patient an inappropriate candidate for this study

Table 1. Eligibility criteria.

Surgical regimen

The regimen of the study is illustrated in **Figure 2**. Prior to patient enrolment, evaluation of the full-thickness burn wounds or of the other full-thickness skin defects was carried out. Preceding the first operation, the full-thickness wounds were treated according to the burn centre's local protocol. Burn depth was initially assessed by means of clinical assessment and later (48h – 5d post burn) confirmed by laser Doppler imaging (LDI) (Moor-LDI-B2, Moor Instruments Ltd, Axminster, Devon, United Kingdom) or with clinical assessment only in case of clear full-thickness burns and assessed by 2 plastic surgeons and/or a burn care coordinator. Other full-thickness skin defects in need of skin grafting were eligible e.g. necrotizing fasciitis, donor site after free radial forearm flap (RFF) harvest and traumatic deep soft tissue injuries (deglovement injuries).

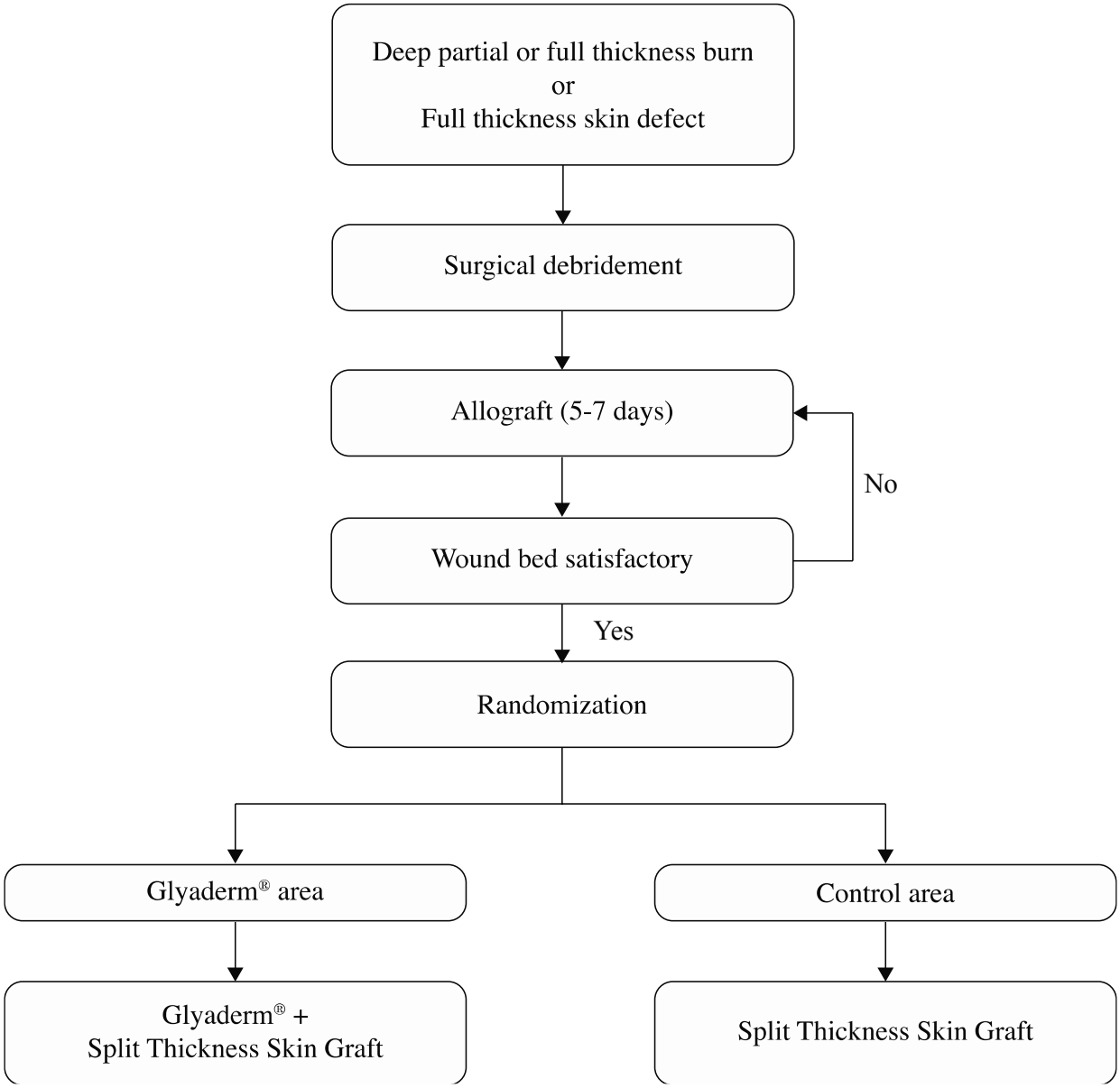

Figure 2. Study flowchart.

The first operation consisted of debridement in combination with the application of glycerol preserved allografts (GPAs, Euro Skin Bank, Beverwijk, The Netherlands) (**Figure 3**) for wound bed preparation. During the second surgical intervention, GPAs were removed and the wound bed was assessed for grafting. If the wound bed was not satisfactory, new GPAs were applied. When deemed suitable for grafting, two comparable wounds or one wound consisting of two comparable parts were randomized into one of the treatment regimens (**Figure 2**).

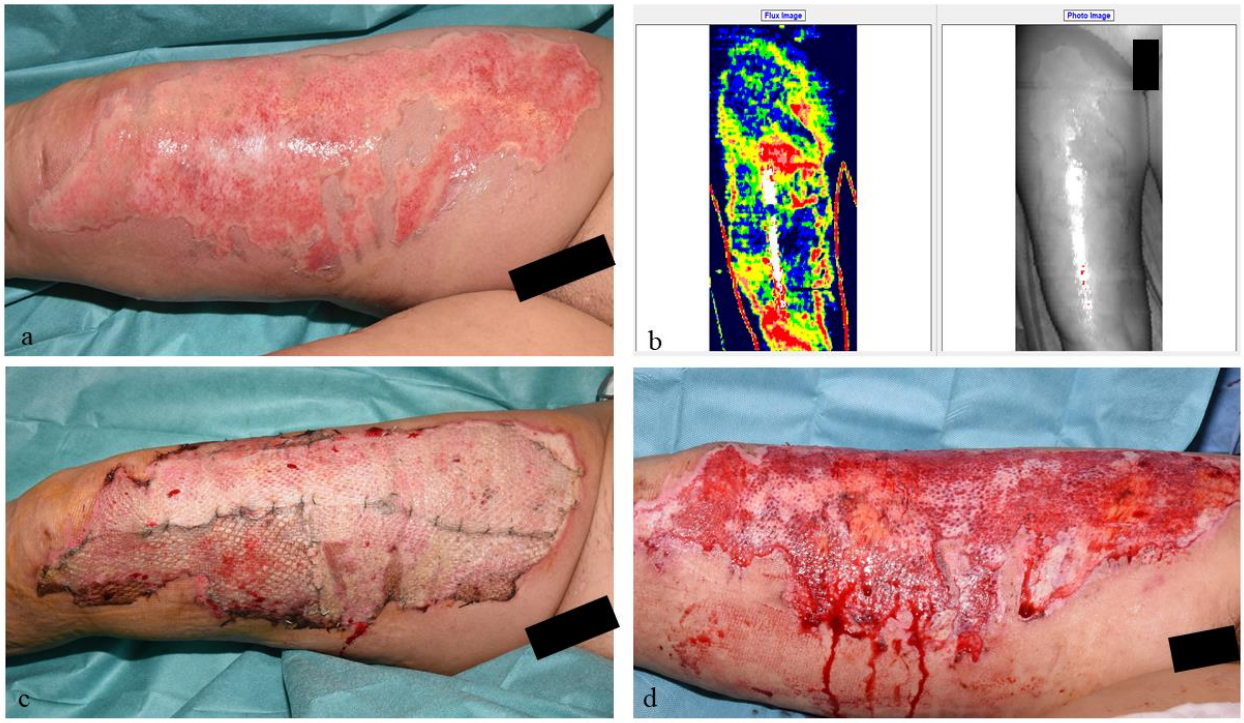

Figure 3. (a) A patient admitted with a scald burn (frying oil). (b) Burn depth assessment by means of LDI on the 3rd-day post-burn. LDI blue colour indicates deep dermal and full-thickness burns. (c) Four days after allograft application. (d) After removal of allografts and prior to application of Glyaderm® and/or autografting.

Wound site selection and randomization

The two comparable wound sites were labelled either A or B prior to randomization. Randomization was performed prior to autografting by use of sealed envelopes indicating the treatment regimen per wound site. Only moments prior to autografting, the sealed envelope was unsealed to reveal the treatment for each site.

Procedure A: Glyaderm® + STSG

The 85% glycerol preserved Glyaderm® was rinsed in sterile water for at least 15 minutes prior to perforation with a special 1:1 ratio carrier (Humeca, Borne, The Netherlands). The Glyaderm® was applied and secured with sutures (**Figure 4**). Subsequently, the Glyaderm® was covered with an autologous STSG (0.012 inches thickness/mesh ratio 1:1,5; 1:2 or 1:3) (**Figure 4**) and secured with staples. In case of phalloplasty donor sites, the autografts were unmeshed and simply perforated using a scalpel. The autograft was then fixed using sutures or staples. The autograft was protected with a semi- permeable membrane: Surfasoft® (Haromed, Ghent, Belgium). The Surfasoft® was covered with a paraffin gauze, povidone-iodine gel, and a sterile gauze.

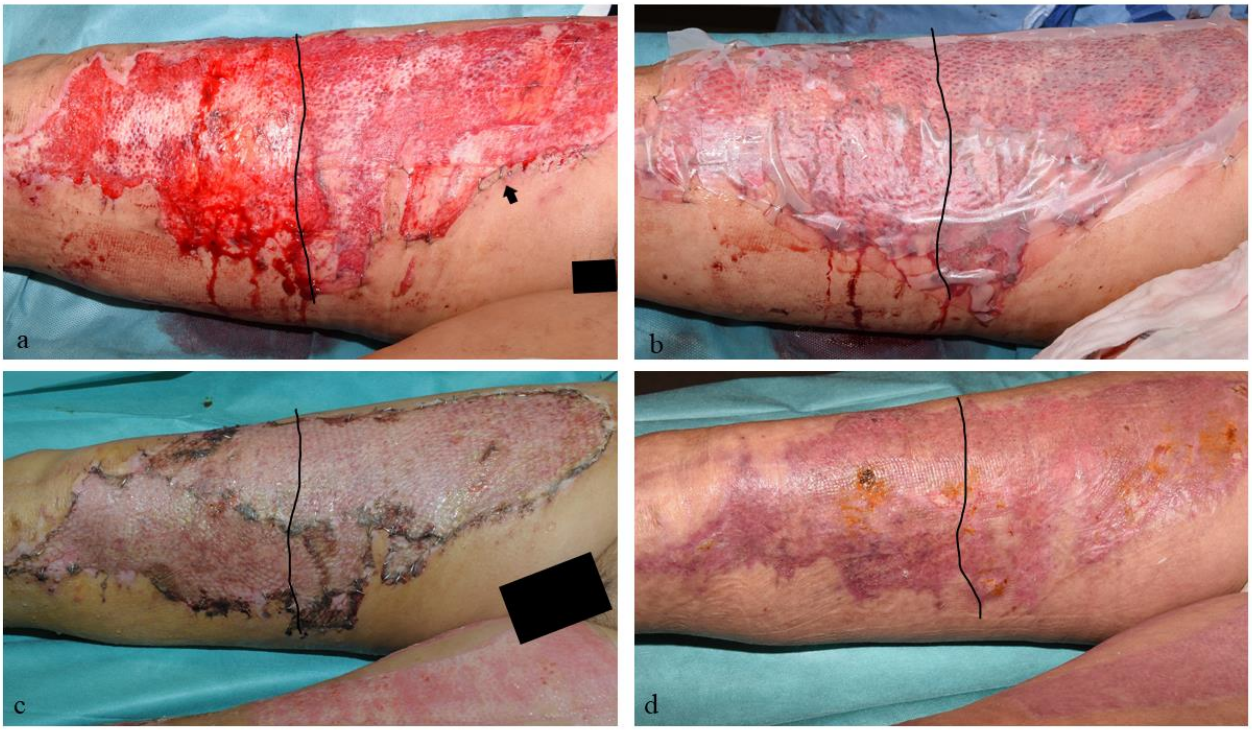

Figure 4. (a) Application and fixation of Glyaderm® on the most proximal half of the upper leg. Control and intervention sites are separated by a black line. Arrow indicates Glyaderm® which can be seen as a thin glistening layer. (b) Autograft application and coverage with Surfasoft®. (c) Removal of Surfasoft® on the 6th-day post-autografting. (d) Complete wound closure 3 weeks post-autografting. 2.7 Procedure B: autograft only. The other wound site was treated with STSG only. The same expansion ratio, fixation methods, and wound dressings were used to ensure comparability between both procedures.

Evaluation during wound healing

Graft take was assessed 7 days post-autografting and scored as a percentage of the total surface area. The pain was assessed at different time intervals ranging from 2 days to 7 weeks post-autografting.

Wound swabs for microbial analysis were performed once weekly. Time until complete wound closure, defined as at least 95 % epithelialization, was registered.

Scar treatment after wound closure

Patients all followed our full treatment schedule consisting of early application of pressure garments (at latest 7-10 days after wound closure), silicones (sheets and garments) and hydration with moisturizers (Alhydran or Dermacress)[14].

Follow-up regimen

The patients were seen at the outpatient clinic for evaluation at 3, 6, 9, and 12 months after wound closure (**Figure 5**). Measurements were taken at all 4 follow-up moments. Elasticity was assessed using the Cutometer. Three parameters were registered: R0, R2, and R8. The R0 value assesses the skin's firmness[32]. The R8

parameter represents the ability of the skin to return to its original state after a deformation[32]. The R2 parameter can be defined as the ratio of these values ($R2 = R8$) and is a $R0$ parameter for elasticity overall[32]. The average of the elasticity measurements of three random sites of each scar area A, B as well as those of normal skin were used. Also, every individual measurement of these 3 measurements per site consists of 3 consecutive measurements, resulting in 1 average value.

Pigmentation and color were assessed using the Mexameter MX 18 (Courage + Khazaka electronic GmbH, Cologne, Germany) with respective parameters erythema index (EI) and melanin index (MI). An average of six measurements all at different sites with the Mexameter was used. Transepidermal Water Loss (TEWL) was assessed by using the Tewameter^{TM} 300 (Courage + Khazaka electronic GmbH, Cologne, Germany). The average of 6 TEWL measurements of two random sites of the scar site as well as those of normal skin was used. Scar hydration was assessed using the Corneometer CM 825 (Courage + Khazaka electronic GmbH, Cologne, Germany). An average of six measurements with the Corneometer, all at different sites, was used. The temperature and humidity of the examination room were always assessed using an ambient condition sensor RHT 100 (Courage + Khazaka electronic GmbH, Köln, Germany).

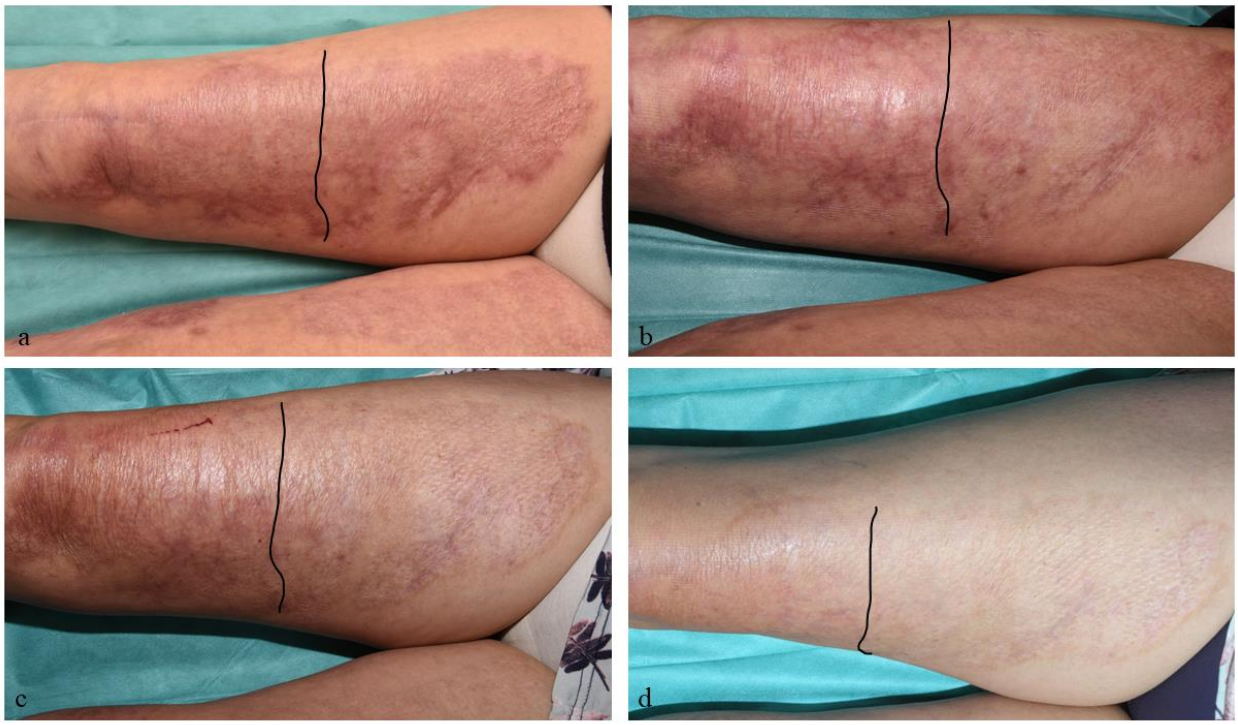

Figure 5. (a) 3 months after wound closure. (b) 6 months after wound closure. (c) 11 months after wound closure. (d) 12 months after wound closure, the site that received Glyaderm® is more supple, has less contracture and the colour is more normalized compared to the control.

Both the Patient and Observer Scar Assessment Scale (POSAS) version 2.0 (Dutch Burns Foundation, Beverwijk, The Netherlands)[33] and the Adapted Vancouver Scar Scale (AVSS) were used to subjectively assess scar quality (Supplementary material 1 & 2 respectively) at

every follow-up. Patients were blinded throughout the study period because they did not know which area was treated with Glyaderm® and STSG and which with STSG alone.

Biopsies

Punch biopsies were taken at 3 and 12 months follow up. Histological analysis was performed by two expert blinded dermatologists (SDS, VV). Automatic Haematoxylin and Eosin staining of the paraffin slices was used (T181 Tissue-Tek Prisma Plus, Sakura Finetek, Antwerp, Belgium). To evaluate the collagen and elastin fiber network, an Elastica von Giesson staining was used (Benchmark special stains, Roche Diagnostics, Diegem, Belgium). The histological slices were stained using Alpha-Smooth Muscle Actin (α-SMA) mouse monoclonal antibodies clone BS66 (Nordic Biosite, Täby, Sweden) to evaluate the number of myofibroblasts (Benchmark Ultra ICH/HIS, Roche Diagnostics, Diegem, Belgium). Biopsies were evaluated in terms of collagen and elastin organization, elastin content and dermal aspect, inflammation including the type of white blood cells, organisation of blood vessels, and number of myofibroblasts. A semi-quantitative scoring system with values ranging from 0-5 was used (see Supplementary material 3). A score of 0 was given to biopsies that resembled normal skin in ECM structure and cellular presence. A score of 5 was attributed to scar tissue with absence of elastin fibers, strong broadened and eosinophilic collagen strings, pronounced dermal inflammation and overall presence of α-SMA. Scores of 1 throughout 4 represent intermediate values.

Statistical analysis

Statistical analysis was performed using Graphpad Prism version 9.0.2 (San Diego, CA, USA). The normality of the data was assessed using the Shapiro-Wilk test. Data are presented as mean ± standard deviation (SD). Pairwise comparisons between two groups with normally and non-normally distributed data were assessed with the paired t-test and Wilcoxon matched-pairs signed-rank test respectively. Pairwise comparisons between more than two groups with normally and non-normally distributed data were detected using the repeated measures one-way analysis of variance (ANOVA) test and Friedman test respectively. A Geisser-Greenhouse correction was applied for the repeated measures one-way ANOVA due to no assumption of data sphericity. Significant differences between groups were followed by a post hoc test. Tukey's and Dunn's multiple comparison tests were used for normally (ANOVA) and non-normally (Friedman) distributed data respectively. A p-value < 0.05 was considered a priori to be statistically significant.

Results

Patients

This clinical trial commenced on the 22nd of February 2017 and ended on the 28th of September 2021. A total of 66 patients were included in this intra-individual study,

corresponding to 82 wound comparisons. Characteristics of the study population can be found in **Table 2** and an overview of the patient recruitment is represented by a Consort flowchart in **Figure 6**. The preponderance of male patients is explained by the relatively high number of phalloplasty donor sites included in the study. These patients were considered as male study participants prior to their gender affirming surgery.

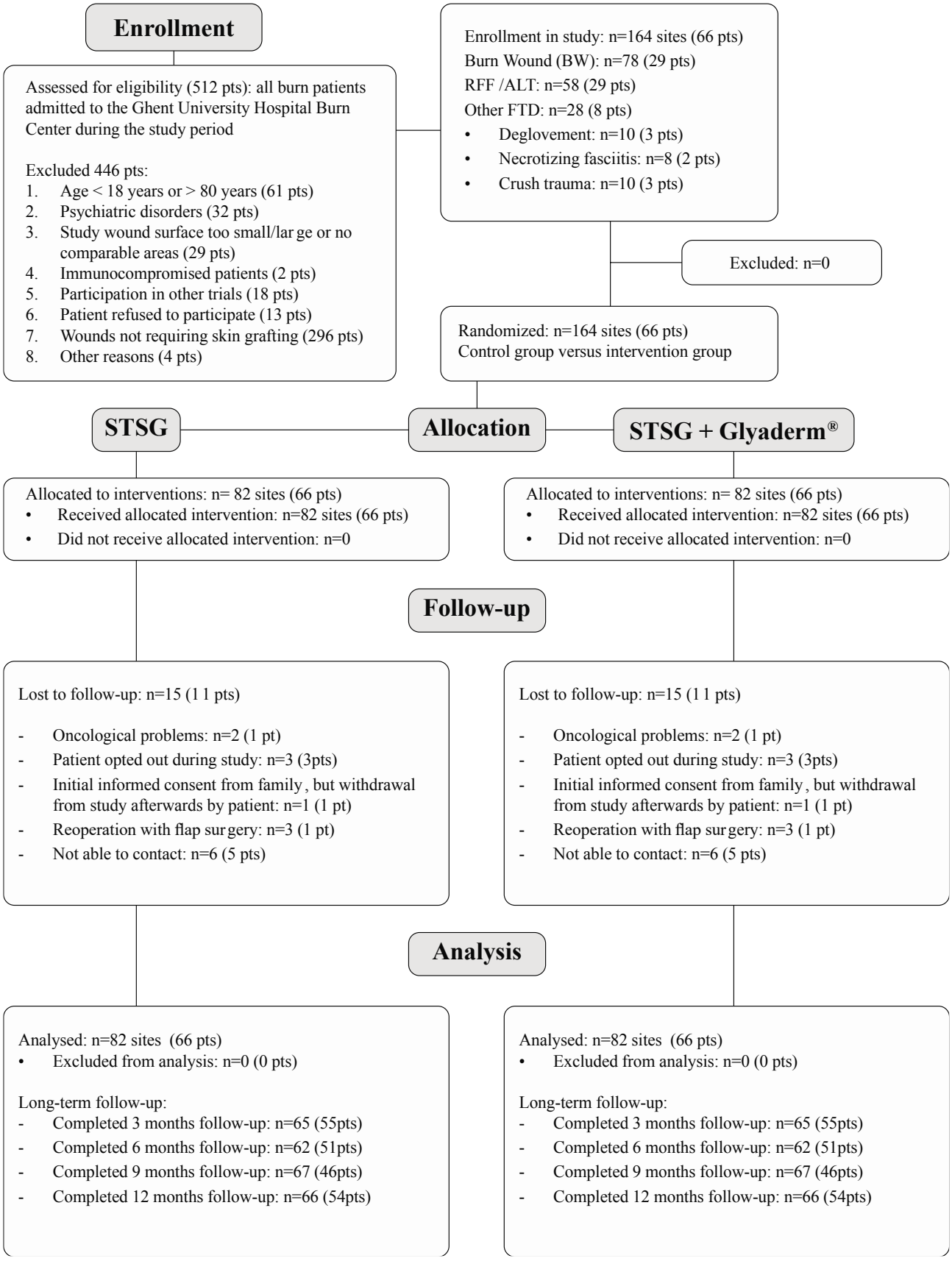

Figure 6. Consort study flowchart of intra-individual study design. Pts = patients. RFF = radial forearm flap donor site. ALT = anterolateral thigh flap donor site. FTD = full-thickness skin defects. STSG = split-thickness skin graft.

Patient characteristics				
Population	Mean (± SD)			
Gender	22 female (44 male)			
Age	39.47 (± 17.99)			
Length	17.94 (± 9.65)			
Weight	76.89 (± 14.68)			
BMI	25.95 (± 4.31)			
Total TBSA	12.33 (± 7.51)			
Patients	WC	Patients	% (WC)	Cumulative %
Total	82	66	100	NA
Burn injuries	39	29	48	48
Phalloplasty donor site ALT|RFF	29	29	35	83
Other full-thickness skin defects	14	8	17	100
EDNX	7	6	9	NA
Expansion rates	Number of wounds	% (WC)	Cumulative %	
Not meshed	30	37	37	
Meshed 1:1.5	26	32	69	
Meshed 1:2	20	24	93	
Meshed 1:3	5	6	99	
Meek 1:3	1	1	100	
Target wounds	Control group Mean (±-SD)		Intervention group Mean (± SD)	
TBSA target wound	2.30 (± 1,87)		2.35 (± 1.94)	
Mean autografts used (cm²)	144.47 (± 112,68)		153.53 (± 118.56)	
Mean Glyaderm® used (cm²)	0.00 (± 0,00)		177.01 (± 127.05)	
Location	Control group	wc	Intervention group	wc
	Foot left	1	Foot right	1
	Gluteal right	1	Gluteal left	1
	Lower arm left	18	Gluteal right	1
	Lower arm right	7	Lower arm left	18
	Lower leg left	8	Lower arm right	7
	Lower leg right	7	Lower leg left	7
	Trunk back	3	Lower leg right	10
	Trunk front	6	Trunk back	3
	Trunk left	1	Trunk front	4
	Upper arm left	6	Trunk right	1
	Upper arm right	5	Upper arm left	8
	Upper leg left	11	Upper arm right	4
	Upper leg right	8	Upper leg left	9
	Total	82	Upper leg right	8
			Total	82

Table 2. Patient characteristics. EDNX = enzymatic debridement with NexoBrid®, RFF = radial forearm flap, ALT = anterolateral thigh flap. WC = wound comparisons, NA = not applicable, TBSA = total body surface area, BMI = Body Mass Index

Evaluation in the acute phase

Pain

The pain was comparable between both groups ($p>0.05$) at every moment of evaluation, except for 5 weeks in favour of the control site ($p=0.031$). Mean pain scores, standard deviations, and statistical tests can be found in Supplementary material 4.

Graft take and time to wound closure

Skin graft expansion rates are listed in **Table 2**. Mean graft take was excellent and comparable in both treatment groups. The graft take was more consistent in the intervention group. Mean graft take was 95.40% (± 10.54) and 96.22% (± 5.40) for the control group and intervention group respectively. No major loss of substitutes or overlying grafts due to inadequate vascularisation or infection was seen. Mean time until complete wound closure was 1.58 (± 0.95) months and was comparable in both groups.

Long-term evaluation of scar quality

Objective measurements

The number of patients, mean values, and corresponding standard deviations of all the objective measurements can be found in Supplementary material 5. The multiple comparisons tests can be found in Supplementary material 6. The used pairwise statistical tests and complementary statistics can be found in **Table 3**

Pairwise comparisons objective measurements																		
			CM TM	Mexameter						Cutometer								
				Erythema			Pigmentation			R0			R2			R8		
Time	Comparison			n	TEST	p-value	n	TEST	p-value	n	TEST	p-value	n	TEST	p-value	n	TEST	p-value
3m	STSG	Glyaderm®		54	Tukey	0,998	54	Dunn's	**<0,0001**$^{-}$	54	Dunn's	0,745	54	Dunn's	>0,9999	54	Dunn's	>0,9999
	STSG	Normal skin				**<0,0001***			**0,0002***			**<0,0001***			**<0,0001**$^{-}$			**<0,0001***
	Glyaderm	Normal Skin				**<0,0001***			>0,9999			**<0,0001***			**0,0008**$^{+}$			**<0,0001***
6m	STSG	Glyaderm®		57	Dunn's	>0,9999	57	Dunn's	>0,9999	56	Dunn's	0,963	NA			56	Dunn's	>0,9999
	STSG	Normal skin				**<0,0001***			**0,002***			**<0,0001***						**<0,0001***
	Glyaderm	Normal Skin				**<0,0001***			**<0,0001***			**<0,0001***						**<0,0001***
9m	STSG	Glyaderm®		52	Dunn's	>0,9999	52	Dunn's	0,842	52	Dunn's	>0,9999	NA			52	Dunn's	0,661
	STSG	Normal skin				**<0,0001***			**0,032***			**<0,0001***						**<0,0001***
	Glyaderm	Normal Skin				**<0,0001***			**0,0009***			**<0,0001***						**<0,0001***
12m	STSG	Glyaderm®	NA	61	Dunn's	0,523	61	Dunn's	>0,9999	58	Dunn's	0,1900	NA			58	Dunn's	>0,9999
	STSG	Normal skin				**<0,0001***			0,0557			**<0,0001***						**<0,0001***
	Glyaderm	Normal Skin				**<0,0001***			**0,033***			**0,0004***						**<0,0001***

Table 3. Pairwise comparisons objective measurements. CM = Corneometer. TW = Tewameter. NA = not applicable due to no significant differences present among groups according to **Table G**. '+' significance in favour of the intervention site. '-' significance in favour of the control site. '*'. significance in favour of normal skin. STSG = split-thickness skin graft. Comparisons concerning the Mexameter control vs intervention group: significant pigmentation/erythema in favour of the control group or intervention group is having an erythema/pigmentation index more in line with the values of normal skin than the other group.

Corneometer CM 825

Mean (±SD) hydration values of SoC, Glyaderm®, and normal skin at 12 months follow-up were 30.40 (±12.43) A.U., 30.91 (±13.75) A.U. and 30.63 (±12.72) A.U. respectively. There were no differences between groups (SoC, Glyaderm®, and normal skin) at 3 months, 6 months, 9 months, and 12 months follow-up.

Tewameter™ 300

Mean (±SD) TEWL values of SoC, Glyaderm® and normal skin at 12 months follow-up were 13.32 (±9.18) g/h/m2, 13.01 (±6.53) g/h/m2 and 13.70 (±6.35) g/h/m2 respectively. There were no differences between groups (SoC, Glyaderm®, and normal skin) at 3 months, 6 months, 9 months, and 12 months follow-up.

Mexameter MX 18

Mean (±SD) EI values of SoC, Glyaderm® and normal skin at 12 months follow-up were 359.14 (±98.64) EI, 368.68 (±86.70) EI, and 291.38 (±84.20) EI respectively. There were no differences between the two treatment groups (SoC and Glyaderm) at 3 months, 6 months, 9 months, and 12 months follow-up. At every follow-up moment, there was a significant difference in EI between treated areas and normal skin.

Mean (±SD) MI values of SoC, Glyaderm® and normal skin at 12 months follow-up were 221.75 (±149.69) MI, 215.68 (±127.37) MI, and 238.50 (±115.18) MI, respectively. At 12 months follow-up there was no significant difference in the control group compared to the intervention group nor was there a difference (borderline) in the control and intervention group compared to the pigmentation values of normal skin ($p=0.056$).

Cutometer MPA 580

Mean (±SD) R0 values of SoC, Glyaderm® and normal skin at 12 months follow-up were 0.63 (±0.35), 0.67 (±0.33), and 0.97 (±0.38) respectively. There were no differences between the control group and intervention group at any follow-up moment. At every moment of follow-up, both the control group and the intervention group had significantly lower R0 values compared to those of normal skin ($p<0.05$).
Mean (±SD) R2 values of SoC, Glyaderm® and normal skin at 12 months follow-up were 0.81 (±0.09), 0.81 (±0.09), and 0.80 (±0.13) respectively. At 3 months follow-up there were no significant differences in R2 values between the control group and intervention group. The R2 values of normal skin were significantly better than those of the control ($p>0.0001$) or intervention group ($p>0.0001$). There were no differences in R2 values between groups (SoC, Glyaderm®, and normal skin) at 6 months, 9 months, and 12 months ($p>0.05$) follow-up.

Mean (±SD) R8 values of SoC, Glyaderm®, and normal skin at 12 months follow-up were 0.51 (±0.25), 0.55 (±0.28), and 0.77 (±0.34) respectively. There were no differences between the control group and intervention group at any follow-up

moment. At every moment of follow-up, both the control group and the intervention group had significantly worse R0 values compared to those of normal skin ($p<0.05$).

Subjective measurements

The number of patients, mean values, and corresponding standard deviations of all the subjective measurements can be found in Supplementary material 7. The used statistical tests and complementary statistics concerning the measurements can be found in **Table 4**.

Subjective measurements																
		3 months				6 months				9 months				12 months			
		n	Test	p-value	Statistic	n	Test	p-value	Statistic	n	Test	p-value	Statistic	n	Test	p-value	Statistic
OBSERVER	Vascularity	62	Wilcoxon	0.122	146	60	Wilcoxon	0.059	195	54	Wilcoxon	0.643	-40	66	Wilcoxon	0.072	178
	Pigmentation	62	Wilcoxon	0.298	107	59	Wilcoxon	0.042^{-}	173	54	Wilcoxon	0.699	33	66	Wilcoxon	0.010^{-}	219
	Thickness	62	Wilcoxon	0.926	17	60	Wilcoxon	0.393	101	54	Wilcoxon	0.094	-110	66	Wilcoxon	0.785	39
	Relief	62	Wilcoxon	0.725	56	60	Wilcoxon	0.297	174	54	Wilcoxon	0.183	-103	66	Wilcoxon	0.330	173
	Pliability	61	Wilcoxon	0.740	-46	59	Wilcoxon	0.065	252	54	Wilcoxon	0.352	-99	66	Wilcoxon	0.131	178
	Surface	62	Wilcoxon	0.933	-13	60	Wilcoxon	0.993	9	54	Wilcoxon	0.930	10	66	Wilcoxon	0.363	91
	Overall opinion	57	Wilcoxon	0.708	52	57	Wilcoxon	0.158	213	54	Wilcoxon	0.336	-79	64	Wilcoxon	0.013^{-}	372
PATIENT	Pain	60	Wilcoxon	0.897	-7	62	Wilcoxon	0.345	-48	55	Wilcoxon	0.881	-7	65	Wilcoxon	0.446	-31
	Itch	60	Wilcoxon	0.321	62	61	Wilcoxon	0.912	8	54	Wilcoxon	0.735	-18	65	Wilcoxon	0.694	-22
	Colour	59	Wilcoxon	0.313	92	61	Wilcoxon	0.158	-136	54	Wilcoxon	0.112	-110	65	Wilcoxon	0.174	-94
	Rigidity	60	Wilcoxon	0.224	107	61	T-test	0.488	0.6977	55	T-test	0.546	0.608	65	Wilcoxon	0.301	116
	Thickness	60	Wilcoxon	0.295	78	61	Wilcoxon	0.318	-101	55	Wilcoxon	0.436	-65	65	Wilcoxon	0.534	-61
	Bumpiness	60	Wilcoxon	0.658	50	62	Wilcoxon	0.331	-129	55	Wilcoxon	0.118	-151	65	Wilcoxon	0.967	-5
	General Impression	60	Wilcoxon	0.393	84	62	Wilcoxon	0.223	-117	55	Wilcoxon	0.005^{+}	-221	65	Wilcoxon	0.029^{+}	-229
AVSS	Color	62	Wilcoxon	0.801	14	62	Wilcoxon	0.471	40	54	Wilcoxon	0.829	-10	66	Wilcoxon	0.610	-37
	Pliability	62	Wilcoxon	0.699	-39	62	Wilcoxon	0.861	-24	54	Wilcoxon	0.951	14	66	Wilcoxon	0.216	116
	Height	62	Wilcoxon	0.590	15	62	Wilcoxon	0.774	13	54	Wilcoxon	0.494	-22	66	Wilcoxon	0.269	35
	Defects	62	Wilcoxon	0.375	-9	62	Wilcoxon	>0.999	2	54	Not possible (*)			64	Wilcoxon	>0.999	-2
	Itch	62	Wilcoxon	>0.999	0	62	Wilcoxon	0.056	44	54	Wilcoxon	>0.999	-2	66	Wilcoxon	0.125	-24
	Pigmentation	62	Wilcoxon	0.745	12	62	Wilcoxon	0.202	52	54	Wilcoxon	0.640	18	66	Wilcoxon	0.782	15
	Total	62	Wilcoxon	0.606	71	62	T-test	0.123	1.565	54	T-test	0.844	0.197	66	T-test	0.957	0.054

Table 4. Pairwise comparisons subjective measurements. (*) Pairwise comparison not possible due to the mean difference between groups being equal to zero. '+' significance in favour of intervention site. '–' significance in favour of the control site.

Adapted Vancouver Scar Scale (AVSS)

No significant differences were found in any of the individual parameters nor the total score of the AVSS at 3, 6, 9, or 12 months follow-up between the control group and the intervention group (p>0.05) (**Table 4**).

Patient and Observer Scar Assessment Acale (POSAS)

POSAS Observer

At 12 months follow-up, all the parameters were comparable in both groups except for pigmentation and the overall score. There was a significant worse score in terms of pigmentation and overall opinion for the intervention group (p=0.010 & p=0.013) (**Table 4**).

POSAS Patient

At both 9 and 12 months follow-up there was a significant difference in terms of overall opinion, in favour of the group that was treated with Glyaderm® (p=0.005 and p=0.013 respectively) (**Figure 7**). The other individual parameters were comparable between the control and intervention group and were comparable at every follow-up moment (**Table 4**).

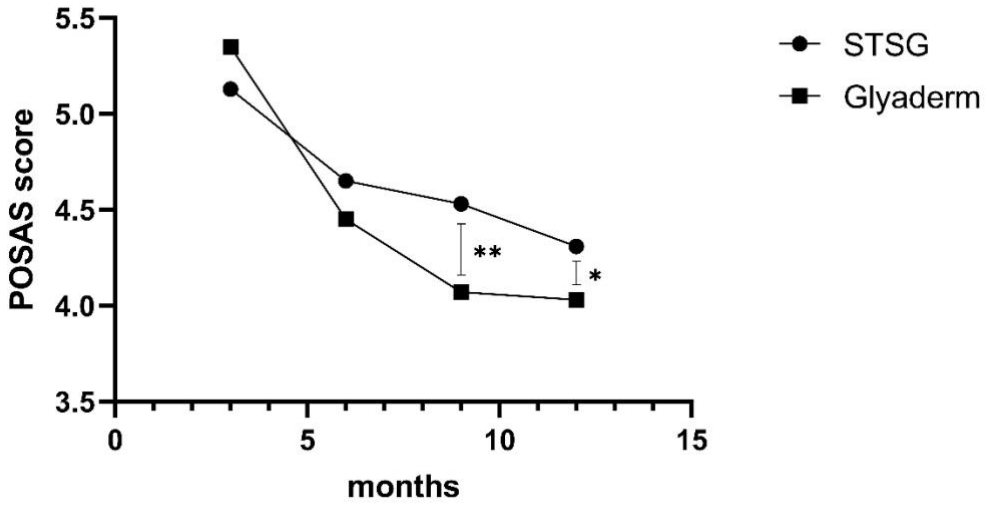

Figure 7. Overall opinion as subjectively attributed by blinded patients using the POSAS at different time intervals during the follow-up period of 1 year after wound closure for both treatments. '*'= significant difference, '**' = strong significant difference.

Biopsies

The number of patients, mean values, corresponding standard deviations and complementary statistics of the histological scores can be found in Supplementary

material 8. No statistically significant differences could be found for the biopsies of the control group versus the intervention group at 3 months and 12 months. However, 57 out of 58 of the sites treated with Glyaderm® clearly showed the presence of donor elastin fibers at 12 months after wound healing (**Figure 8** (f)), illustrating the longevity of the fibers. Presence of elastin was characterized by a histological score of 4 or less. A satisfying number of elastin fibers of favourable quality, characterized by a histological score of 3 or less, were seen in 34 out of 58 biopsies.

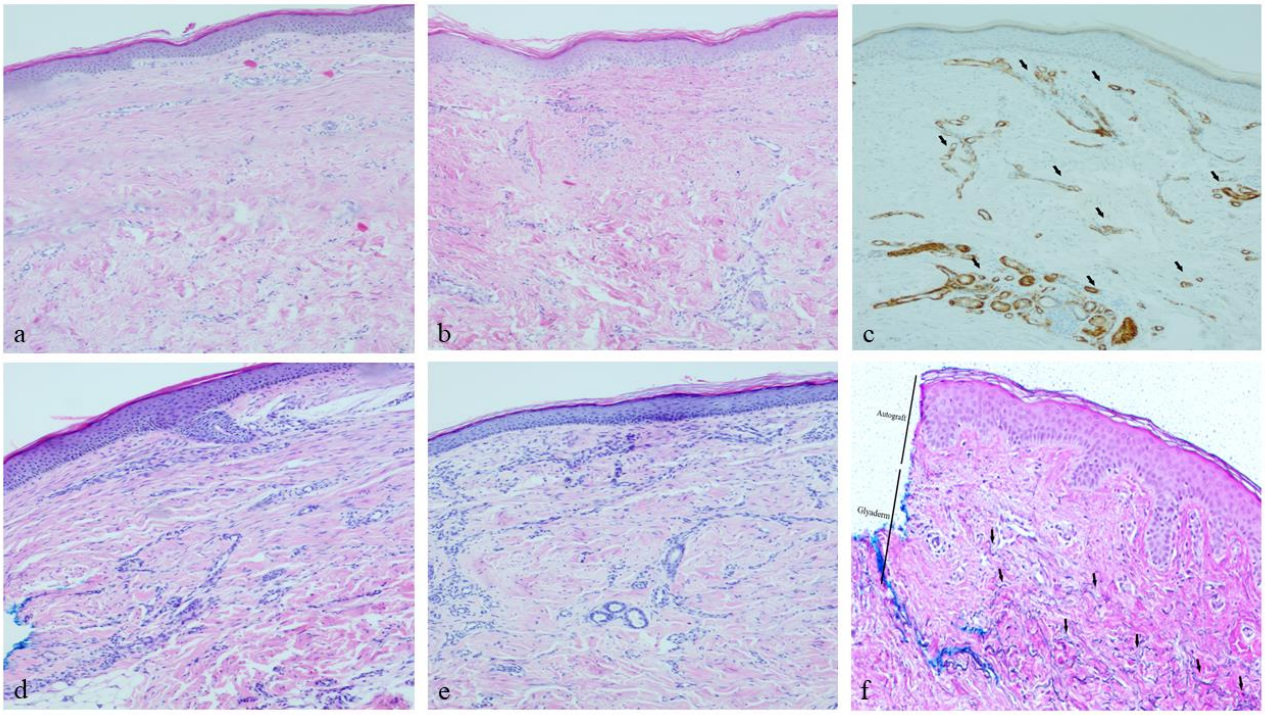

Figure 8. Light microscopy of histological slices - (a) HE histological slice of control site at 3 months follow-up. (b) HE histological slice of intervention site at 3 months follow-up. (c) α-SMA staining of histological slice of the intervention group at 3 months follow-up. Arrows indicate vascular structures in the papillar and reticular dermis indicating a well-vascularized neo-dermis. (d) HE histological slice of control site at 12 months follow-up. (e) HE histological slice of intervention site at 12 months follow-up. (f) Elastica Von Giesson staining visualizing the presence of donor elastin fibers at 12 months follow-up. Autograft and Glyaderm® are indicated with two-sided arrows. Single arrows indicate some example donor elastin fibers in the deep dermis. The elastic fibers are coloured blue due to the staining procedure.

Discussion

Early debridement and immediate coverage of extensive skin defects with STSG is essential for the survival of patients with severe burns but the lack of dermis often results in HTS formation and contractures[16]. In case of extensive full-thickness burn wounds, the dermal layer cannot be reconstructed using the classical reconstructive approaches such as full-thickness skin grafts or flaps. A possible alternative is to use dermal substitutes with immediate or delayed autografting[34]. In this study, we

investigated the short- and long-term cosmetic and functional outcomes following the use of Glyaderm® in a single-stage procedure.

The major advantage of acellular dermal templates derived from human allograft skin such as Glyaderm® is that they retain the native dermal structure, with the characteristics of the natural porosities required for dermal regeneration, vascularisation and innervation[18,24,35]. When combining a dermal substitute and autograft in a single operation, the main limiting factor is inadequate vascularisation, risking partial or complete necrosis of both, substitute and autograft[36]. Most DRT's are applied in a 2 step procedure, with autografting being delayed for several weeks to ensure incorporation and vascularization of the inherently avascular substitute[7]. However, the literature confirms that dermal replacement and coverage with skin grafts, primarily due to shrinking health care resources, should ideally be performed in a single-stage procedure if autografts are available[37]. In general the thickness of DRTs ranges from 0.040–0.080 inches (1-2 mm)[34]. The restricted and standardized thickness of Glyaderm® (0.012 inches or 0.30 mm) grants fast and adequate neovascularization and allows a one- stage procedure, illustrated by the excellent average graft take of 96.22%, combined with only a limited standard deviation (±5.40), achieved in this study[34].

Research has shown that alterations in both elastin organization and content contribute to the formation of scars[23]. A key component of Glyaderm® is the natural collagen-elastin matrix in which elastin fibers with microfibrils are incorporated and well-preserved even after decellularization[25,26,38]. The incorporation of elastin either acts as a replacement or potentially promotes the synthesis of elastin fibers as is seen in animal models, where the use of these collagen-elastin scaffolds can even induce a limited level of elastin fiber deposition, whereas collagen-only scaffolds do not[18,24,39,40]. The presence of elastin interrupts myofibroblast differentiation and therefore less collagen contraction is observed, leading to improved elasticity of the scar[1]. By means of the skin biopsies performed at 3 and 12 months, this study demonstrates the longevity of the transplanted donor elastin fibers and improvement of scar elasticity was shown with improved overall opinion on the patient POSAS. The collagen fibers present in xenografts or synthetic grafts have often been chemically cross-linked to enhance stability and decrease susceptibility to early degradation[16,4]. Expedited degradation is unfavourable due to the potential risk of increased fibrosis[42]. However, early degradation is not desirable, but having no implant degradation can impede cellular activity in situ[42]. Due to the cytotoxicity of the cross-linking chemicals, adverse effects on host response might be considerable[16,41]. In contrast, non-cross-linked templates, such as Glyaderm®, are well tolerated and stimulate tissue regeneration in addition to minimal inflammatory responses, whilst still respecting one of the main principles of reconstructive surgery: replacing '*like with like*'[16,19,41].

Probably most remarkable in this study is that none of the Glyaderm® dermal substitutes was lost due to major infections, inadequate vascularization nor due to other complications. The price of the two most well-known collagen-elastin acellular dermal matrices (ADMs) the human derived Alloderm® and bovine derived

Matriderm is respectively €30/cm^2 [43] price 2012) and (0.080 inches – 2 mm) €5.02/cm^2 (price 2022)[44–46]. Alloderm® can be used in a single stage and is mostly used for soft tissue augmentation around implants, dental root coverage and gingival augmentation. Its use is limited in the reconstruction of burn injuries. Glyaderm® costs €4.74/cm^2 (price 2022) and has lower costs compared to other biological collagen-elastin acellular dermal matrices[44]. Prices in this article were obtained through representatives with the exception of the price of Alloderm®, which was obtained through literature. All given prices are target prices and depend on e.g. order quantity, substitute dimensions, and the hospital. However, the best known and probably most widely used DRT is Integra, an approximately 0.030 inches (0.80 mm - €16.36 /cm^2 price 2022) thick bilaminar cross-linked bovine derived collagen based dermal matrix requiring a two-stage procedure[1,4,47]. The necessity of a two-stage procedure, high risk of infection, inconsistent long term results, absence of elastin and the huge financial burden are the most reported drawbacks and are therefore important limiting factors for general use[4,47]. The problem with synthetic bilayers is the difficult initial wound adherence and fluid accumulation which leads to the development of seromas and harbours an increased risk for infection, which is the most frequent complication seen with Integra® [35,47]. A recent paper published by Gonzalez et al. reviewed 26 studies reporting infection rates with the use of Integra®[47]. The research group stated that on average 16.9% of Integra engrafted sites led to infection[47]. In this study, none of the Glyaderm® nor the covering autografts were lost due to (major) infection. Although the two stage technique is deemed to be reliable, it also necessitates a treatment period that is prolonged with several weeks to allow sufficient ingrowth of supporting blood vessels, requires additional operations and anaesthetic administrations[7]. Additionally a two-staged technique is subsequently associated with increased hospitalization time and a higher number of outpatient visits[7]. However, when confronted with limited availability of donor sites, temporary coverage by dermal substitutes in a two-stage procedure can be beneficial.

The substantial reductions in hospital length of stay, less operative encounters, reduced outpatients visits and health-care expenditures combined with the fact that infections might lead to potential loss of both the dermal substitute and covering graft, a significant decrease in costs can be expected when using dermal replacements making use of a single-stage engraftment[7]. However, not all dermal substitutes allow for a successful one-stage procedure and require multiple surgeries for reconstruction. Integra Single layer 'Thin', is a 0.016 inches (0.40mm – €12.2/cm^2 price 2022) thick DRT that can be used in a one stage setting, but lacks elastin fibers. Furthermore, bovine derived Matriderm 1mm single layer (€5.32/cm^2 (price 2022)) and human derived SureDerm® (price not available) are two collagen-elastin DRT's commercially available of being used in a one-stage setting. Limitation of health resources have made the need for comparative studies between commercially available DRTs even more crucial and should be subject to future trials [48].

In this single-blinded, randomized controlled trial, our research group demonstrated the successful applicability of simultaneous bilayered skin reconstruction using Glyaderm® as an acellular dermal substitute in patients with various full thickness

defects. There was no loss of Glyaderm® due to inadequate vascularization nor infection, associated with nearly perfect graft take, comparable wound closure times. Although the thickness of Glyaderm® has been standardized and restricted to a thin 0.012 inch sheet, which is much thinner than most dermal substitutes, this acellular DRT led to increased patient satisfaction based on the POSAS (overall opinion) of the patient[34]. The majority of patients indicated that these more favourable results are due to a more normalized skin sensation at the site that received dermal replacement. Decreased donor site morbidity by preserved sensory functioning has been reported with the use of dermal substitutes covering phalloplasty donor sites[49]. Watfa et al. investigated the effects of single stage reconstruction with MatriDerm® after radial forearm flap harvest and found that the group that was treated with the bilayered skin reconstruction had more preserved sensory nerve functioning and skin sensibility[49].

Compared to the two-staged procedure, a one stage-reconstruction with Glyaderm® did not deliver a statistically significant improvement in terms of scar elasticity[24]. However, the reduced thickness of the Glyaderm® sheet may provide less benefit in elasticity compared to a more substantial layer[37].

The ideal skin substitute should be inexpensive, effective, widely available, easy to produce, easy to transport and store, of humane origin, should have a low infection susceptibility, should lack antigenicity, should quickly adhere to the wound bed, should protect the wound from dehydration, should allow excellent graft take, should activate and modulate the cicatrisation process, should not be biodegradable too quickly and should finally but most importantly result in improved scar quality[17,50]. The results of previous extensive research combined with the outcomes of this high level evidence study are favourable towards presuming that Glyaderm® is an ADM that meets most of these rigorous requirements[24-26,44,51,52].

Limitations of the study

Part of the study period was during the COVID-19 pandemic and thus some patients could not receive their proper follow-up leading to loss of valuable data. To counter this loss, the research group decided to include additional patients, raising the number of wound comparisons from the included 75 to 82.

Conclusions

Combined with adequate debridement and proper wound bed preparation, a standardized thickness of 0.012-inch Glyaderm® enables the use of a cost-effective single-stage procedure for deep and full-thickness skin defects, which is universally favoured by all surgeons. In contrast to most dermal substitutes available, no infections were seen and optimal graft take was achieved. Glyaderm® can thus be used as an acellular dermal matrix in the reconstruction of full-thickness burns or other comparable full-thickness defects, eventually resulting in long-term increased patient satisfaction and therefore QoL.

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CHAPTER 11

FINAL UPDATE ON THE CLINICAL APPLICATION OF GLYADERM® AND ITS SCOPE

JOURNAL OF PLASTIC, RECONSTRUCTIVE & AESTHETIC SURGERY (SUBMITTED IN PART)

A. PIRAYESH
I. DE DECKER
H. HOEKSEMA
C.D. RICHTERS
M.J. HOEKSTRA
L. GUERRERO
J. VANOORBEEK
K.E.Y. CLAES
B. VAN DER LEI
S. MONSTREY

Introduction

Burn injuries represent one of the top causes of injury-related death, and its incidence varies worldwide[1]. Significant advancements in burn care made over the past decades have led to improved patient survival and recovery, shifting the primary goal of management of severe burns from mere survival towards improving the "quality" of patient survival[2,3]. Quality of life largely depends on how patients re-integrate into society whereby the scar quality and its appearance, and the perception of their own appearance play a very important role. Autologous split thickness skin graft (STSG) is the current gold standard for the treatment of deep dermal and full thickness burn wounds[3,4], however, there are numerous challenges associated with STSGs, including limited donor site availability, donor site morbidity, graft contracture, and an unpredictable or sometimes poor scarring process[5,6]. In addition to human allografts, epidermal and/or dermal biologic, and synthetic skin substitutes have emerged in the last few decades also as a possible way to improve scarring after burn injury[5].

Dermal regenerative matrices (DRMs) are permanent skin substitutes used allowing regeneration of the dermal skin component in the management of full thickness skin defects left after excision of burn wounds or release of burn wound contractures. DRM is approved for use in acute burn surgery and burn reconstruction[7] and has been shown to produce excellent functional and aesthetic results for both indications[6,8–12]. In addition to its many benefits, DRM has also been cited to have several disadvantages, including the need for a two-stage procedure[13–15], increased infection risk[13,15], and high cost[12,15].

Within the last 20 years we set out to develop "Glyaderm®" collagen-elastin matrix DRM derived from glycerol preserved allograft which has been reported in animal studies and clinical trials (**Table 1**). This overview is an update on scope (**Table 2**) and the clinical application of Glyaderm® DRM.

Milestones Glyaderm® DRM	Year	Outcome	Publication
Formation research group	2001	Start Glyaderm® studies in porcine model	
Animal studies burn wounds	2003	Favorable results of Glyaderm® compared with different dermal substitute matrices in a porcine wound model	JPRAS[16]
First clinical case	2005	Full take of Glyaderm® on neck treated at Gent Burn Centre	
First publication	2008	DRM Feasible from glycerol preserved allograft treated with NAOH	Cell Tissue Banking[17]
Animal studies abdominal fascia	2008	Glyaderm® may be used in clinical trials for closure of abdominal wall defects.	European Surgical Research[18]
Patent IP Glyaderm® to EuroTissueBank by Research Team	2009	Research group allows patent to EuroTissueBank for Non-Commercial distribution	
First case breast reconstruction	2013	Glyaderm® DRM can be used for full implant coverage	
One stage & two stage procedure 55 patients	2014	Glyaderm® dermal substitute: Clinical application and long-term results in 55 patients	Burns[19]
Proof of native collagen elastin matrix	2015	Visualization of newly synthesized collagen *in vitro* and *in vivo*	Scientific Reports (Nature)[20]
Full Face reconstruction ACID Attack case	2015	Glyaderm® dermal substitute introduced to Colombian Plastic Surgeons	
Glyaderm® to cover exposed bone	2016	Exposed tibial bone after burns: Flap reconstruction versus dermal substitute	Burns[21]
Glyaderm® produced in Colombia	2017	SkinBank in Bogota produces Glyaderm® in collaboration with EuroTissueBank	
Glyaderm® combined with stem cells	2019	Glyaderm® effective carrier for ASCs in full-thickness wounds. ASC-seeded Glyaderm® significantly enhanced wound healing	Ann Med Surg[22]
One Stage l procedure randomized trial in 66 patients	2023	Prospective, randomized, controlled, blinded study of simultaneous application of Glyaderm® +STSG gives superior patient satisfaction vs STSG	Burns & Trauma[23]

Table 1. Overview of developments and studies, scopes and clinical applications of Glyaderm® DRM.

Historical Aspects

At the start of the millennium our research group was faced with challenges in burn care reconstruction. The DRM Integra was gaining attention in literature and at conferences but the variability in take rate and high cost prevented its widespread application and routine use in the Gent Burn Unit.

Our group had been conducting laboratory and animal studies with cultured keratinocytes and various dermal matrices with limited results.

The collaborative brainstorming sessions culminated in the idea of developing a dermal substitute based on glycerol preserved allograft which would be a non-commercial and cost-effective DRM for widespread application and improvement of the quality of life of the burn patients. Glycerol preserved allograft was developed by Hoekstra and co-workers and distributed in 20 European countries by then EuroSkinBank now EuroTissuebank.[23]

Pirayesh, Hoeksema, Richters, Hoekstra and Monstrey signed away their IP rights for the DRM "Glyaderm®" which was affirmed by notary to the EuroSkinBank, now EuroTissueBank, Beverwijk, The Netherlands.

Burn reconstruction (deep dermal & full thickness)
Burn Scar reconstruction
Facial Acid Attack
Soft tissue defect reconstruction after trauma or oncological resection
Post necrotizing fasciitis
Giant melanocytic nevi
Donor Site reconstruction (Radial forearm phalloplasty)
DRM for Breast reconstruction

Table 2. Scope of Glyaderm® DRM

Introduction

Glyaderm®

Glyaderm® is a collagen-elastin DRM resulting from processing glycerol preserved allogenic donor skin.

Application

Operative procedure

The 85% glycerol preserved Glyaderm® is rinsed in sterile water for at least 15 minutes prior to perforation with a special 1:1 ratio carrier (Humeca, Borne, The Netherlands). The Glyaderm® is applied and secured with sutures (**Figure 1**).

Subsequently, the Glyaderm® is covered with an autologous STSG (0.012 inches thickness/mesh ratio 1:1,5; 1:2 or 1:3) (**Figure 1**) and secured with staples. The autograft is then fixated using sutures or staples. The autograft is protected with a semi-permeable membrane: Surfasoft® (Haromed, Ghent, Belgium). The Surfasoft® is covered with a paraffin gauze, povidone-iodine gel, and a sterile gauze.

Post-operative regimen

Wound assessment is performed at day 5-7 after which the Surfasoft® layer is removed Wounds with adequate take rate are treated with pressure garments and Aloe Vera based scar cream as standard of care at our unit.

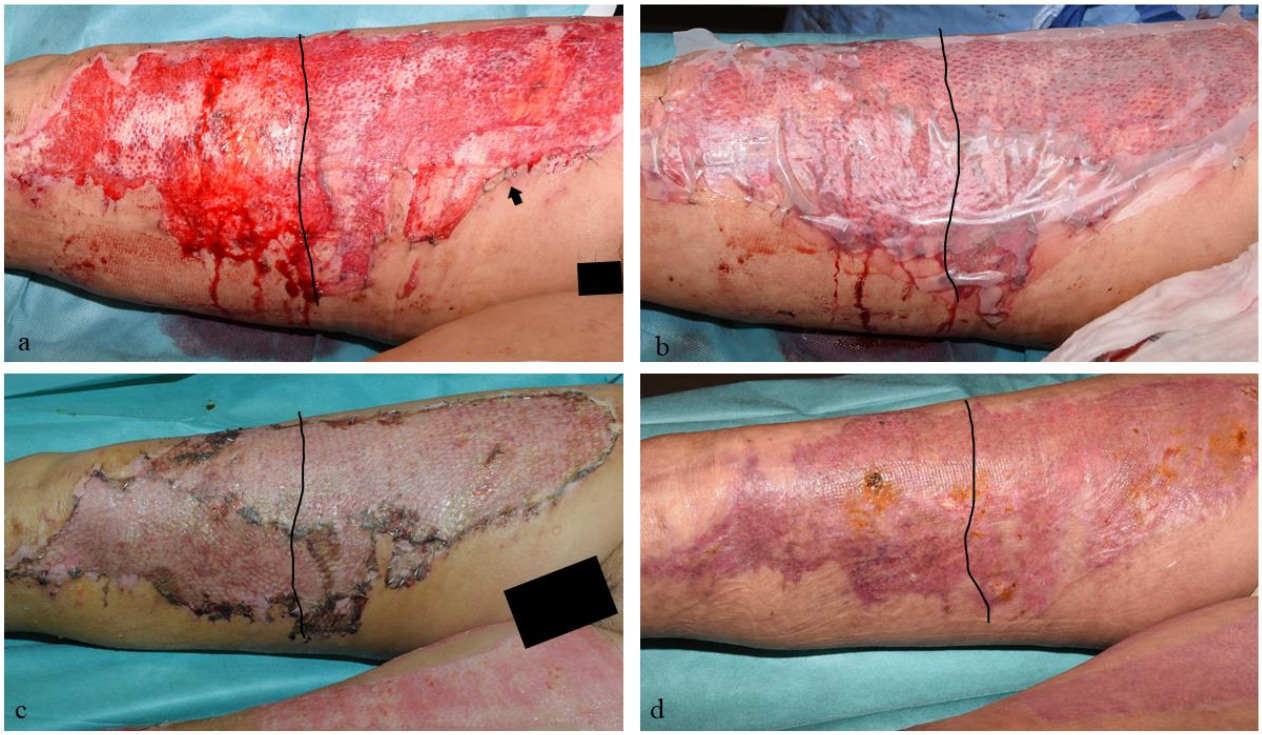
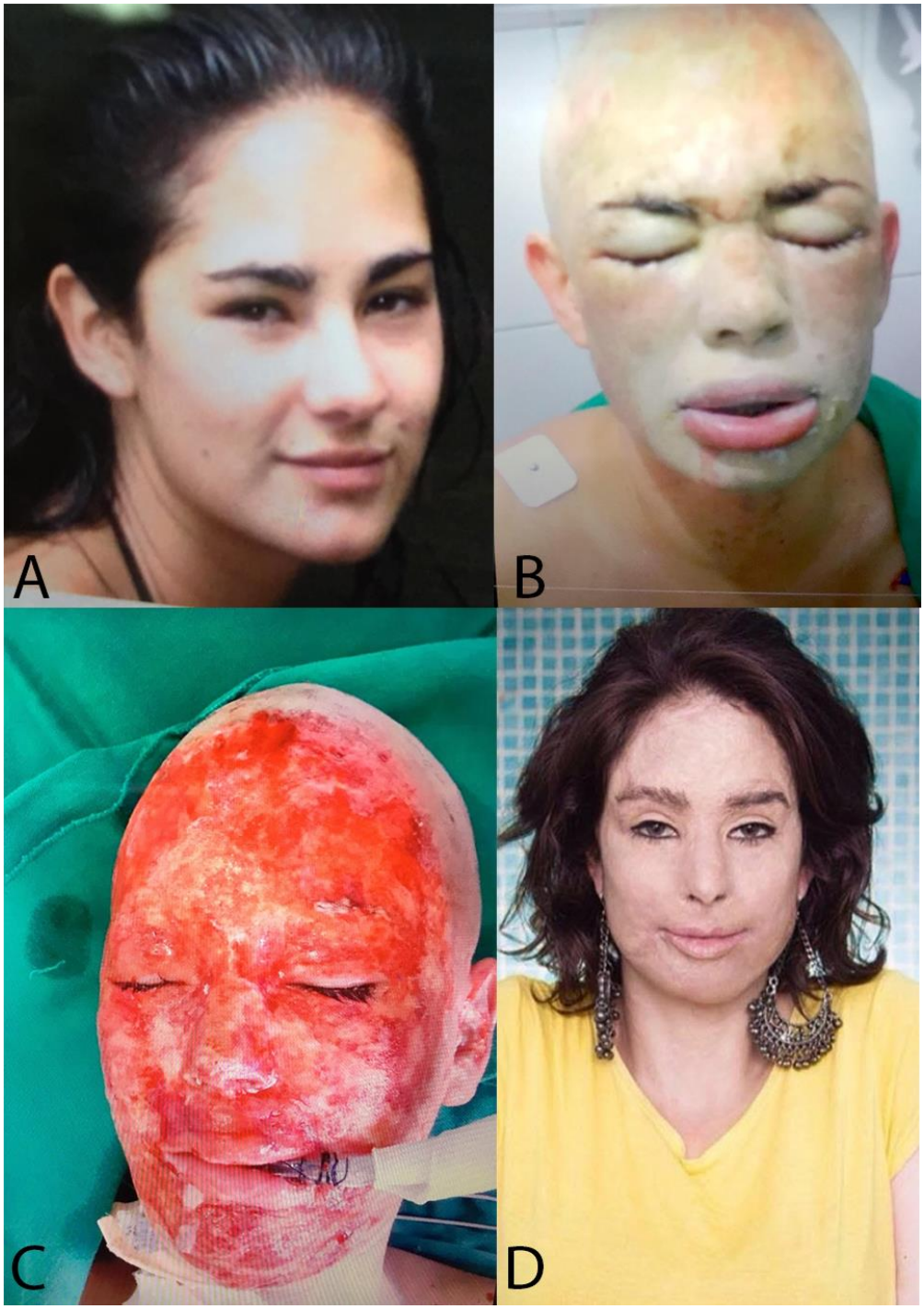


Figure 1. (a) Application and fixation of Glyaderm® on the most proximal half of the upper leg. Control and intervention sites are separated by a black line. Arrow indicates Glyaderm® which can be seen as a thin glistening layer. **(b)** Autograft application and coverage with Surfasoft®. **(c)** Removal of Surfasoft® on the 6th-day post-autografting. (**d)** Complete wound closure 3 weeks post-autografting.

Case studies

Case Study 1. 20-year-old woman with full thickness burn after Acid Attack

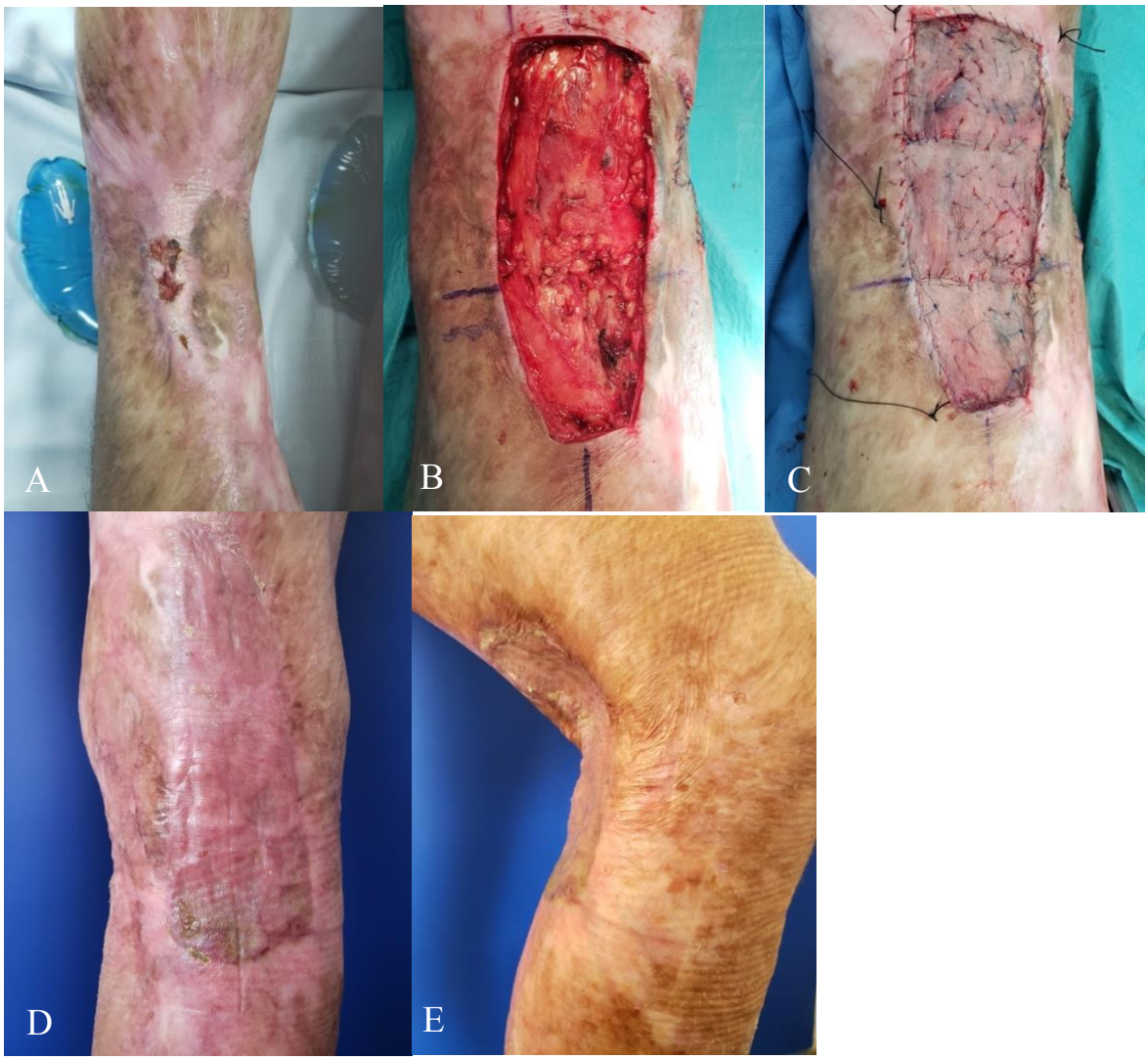
A. Pre-operative
B. Full thickness chemical burn
C. After debridement
D. 2 years after Glyaderm® DRM and SSG and lipofilling, PRP and laser resurfacing[25]



Case Study 2. Burn Scar Contracture treated with Colombian SKINBANK Glyaderm® one Stage in Bogota

30-year-old woman with a full thickness constricted unstable burn scar with limited range of movement at right elbow joint

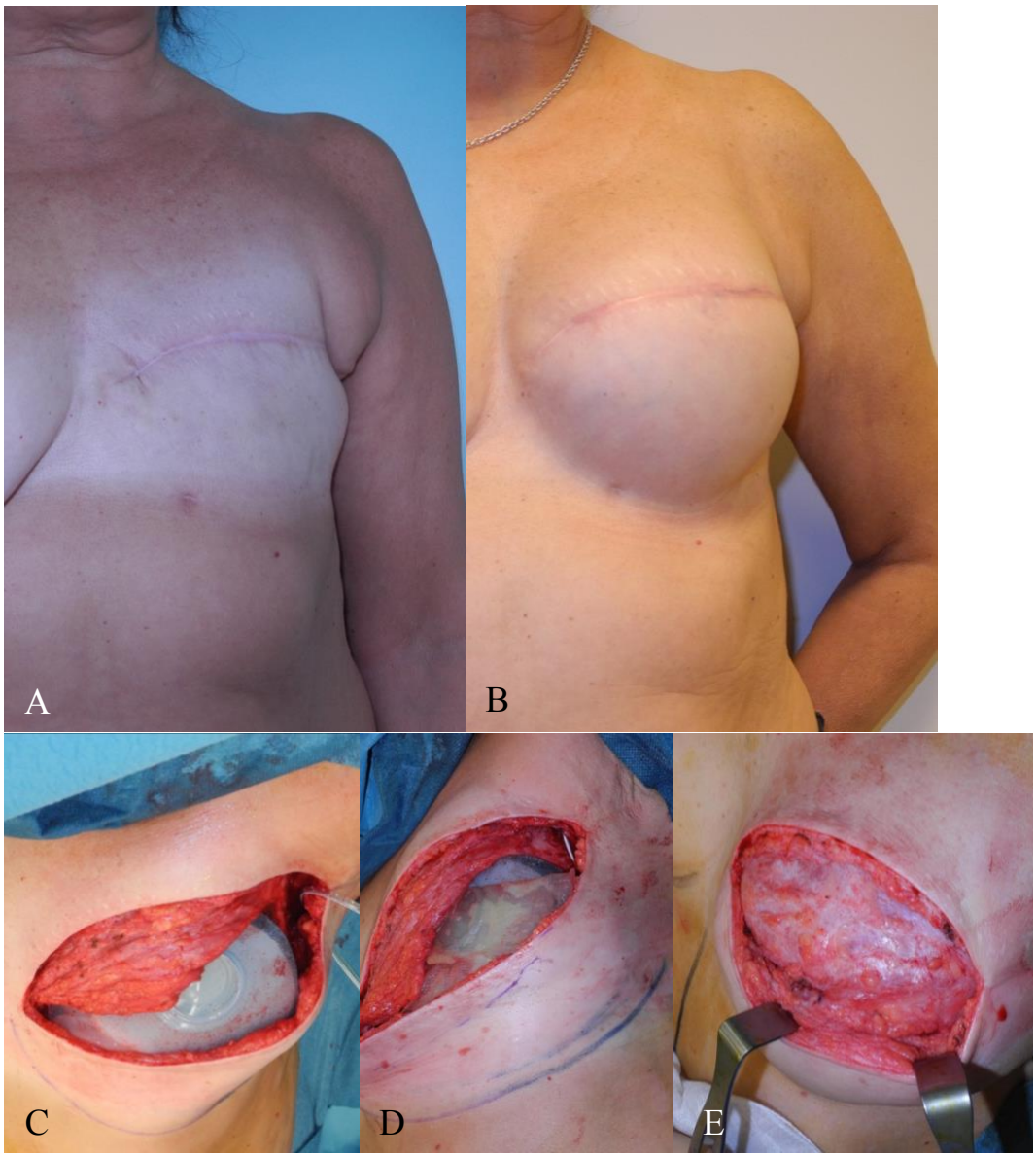
A. Pre-operative constricted burn scar.
B. Radical Surgical Debridement
C. One - Stage Glyaderm® and SSG with quilting sutures
D. Stable bi-layered restoration at 6 months
E. Improved sable range of movement at elbow joint



Case Study 3. Breast reconstruction

50-year-old woman with primary breast cancer

A. Pre-operative after mastectomy
B. Postoperative after reconstruction with total implant coverage with Glyaderm®
C. Tissue expander
D. Subpectoral tissue expander covered with lower pole Glyaderm®
E. Total implant coverage possible after ingrowth of Glyaderm®



Dermal Substitutes

The standard procedure for treating full thickness skin defects and deep burns consists of debridement of the necrotic tissue and coverage with an autologous split thickness skin graft (STSG)[26,27]. STSG consists of epidermis and a thin layer of dermis. Expansion of the graft can be achieved by meshing.

Meshing creates small perforations in the skin, allowing the STSG to expand through stretching. The interstices created by this method will heal from the graft margins, leading to reepithelialisation, possibly prolonged granulation tissue formation, which in turn can result in a longer healing process and increased scarring[28]. Wounds treated with meshed grafts are more likely to show unfavourable scarring compared to unmeshed STSG[29].

It is possible to use an autologous full thickness skin graft (FTSG) to replace both the epidermis and dermis, but due to the paucity of the donor sites (i.e., groin, post-auricular, lower abdomen) this is not really an option in extensively full thickness defects[30]. A more universal method to replace the dermis is available (i.e. dermal substitutes). The lack of dermal tissue in full thickness skin defects and the reduced quality of the scars after treatment with STSG or CEA, which contain little or no dermal component respectively, initiated the development of dermal substitutes[24,29].

Using dermal substitutes has distinct advantages. A dermal substitute is a replacement, without the need for removal afterwards. Most importantly, it provides a scaffold allowing the ingrowth of blood vessels and the influx of cells which will lead to the formation of a neo-dermis. The dermal substitute acts as a support for the new cells.

The migration and proliferation of these new cells can take place in an organised manner, leading to a high degree of tissue regeneration and wound closure[31]. The STSG used for subsequent grafting can be thin, because there is already a dermal layer present. The donor site will heal faster, resulting in less donor site morbidity which is often underestimated, and providing new transplantable skin sooner if needed[31].

Based on the desired functions of the dermal substitute, certain properties are required:

1. Prevention of microbial entry, overheating and fluid loss/accumulation[24,26,28,29,33]
2. Stability, biodegradation and immunocompatibility[33,34]
3. Host or enable the influx of cells that will function as dermal cells[33]

Other desired characteristics of a dermal substitute include:

1. Resistance to shear forces[24,29,32,33,35]
2. Cost-effective and widely available[35,36]
3. Optimal application on both regular and irregular wound surfaces[35]
4. Easy to prepare, store and use[36]
5. Sterility[34]
6. Able to withstand wound hypoxia[36]

The following commercialised products are popular examples of dermal substitutes.

Integra®

Integra® is a dermal substitute based on bovine collagen and chondroitin-6-sulfate (derived from shark cartilage)[37]. The matrix is chemically cross-linked to counteract early degradation of collagen[30]. It has been approved for the treatment of burn injuries in 1997[26]. This dermal matrix is covered with a silicone layer, providing instant wound coverage after application. The silicon layer is removed when the substitute is adequately vascularised, which will take approximately three weeks[37]. More recently Integra® dermal regeneration template single layer (IDRT-SL) has become available. It is a specialized dermal substitute which can be applied during the same procedure as the autografts, making it a one-stage procedure. In the treatment of deep wounds, it can even be combined with 'normal' Integra® for a two-stage procedure.

Matriderm®

Matriderm® is a decellularized dermal substitute based on bovine collagen, that has been coated with elastin hydrolysate[30]. Matriderm® is non-cross-linked making it bioresorbable. Matriderm® has been used successfully to treat full-thickness skin defects[27]. Matriderm has been successfully used in a single-stage procedure.

Alloderm®

Alloderm® is a decellularized dermal substitute derived from human cadaveric skin. It lacks an epithelial component, but it is possible to graft a STSG during the same procedure[26]. Alloderm® is well tolerated by patients and has been used to treat minor defects. Alloderm® has a possible risk of disease transmission[38].

Glyaderm®

Glyaderm® is the result of processing glycerol preserved allogenic donor skin. Using glycerol has some distinct advantages. Skin preservation using glycerol is not only cheaper than cryopreservation, GPA is also less immunogenic. Glycerol has bactericide properties; 97% of bacteriologic cultures from GPA are negative after 3 months[39]. Glycerol can inactivate viruses such as HIV-1 and Herpes Simplex[40]. The donor cells are non-viable due to the glycerol preservation method, but the collagen and elastin networks remain intact. In Glyaderm® all the donor cells (i.e. hair cells, vascular endothelium, smooth muscle, keratinocytes) must be removed in order to avoid an adverse inflammatory (immunogenic) response resulting in the rejection of the skin replacement[30]. The main advantage of using a GPA-derived dermal substitute is that it resembles the natural collagen-elastin native structure of the human skin. This contrasts with other substitutes, such as Matriderm® or Integra® which are from animal and or synthetic origin. It is essential to preserve the natural collagen and

elastin 3D fibre network of the dermis. A few years ago, a panel of experts stated: "Given current knowledge, the ideal acellular matrix is one that most closely approximates the structure and function of the native extracellular matrix (ECM) it is replacing"[31]. The glycerol is removed by rinsing the GPA in a sterile saline (NaCl) solution. Washing is done repeatedly, ensuring that residual glycerol is removed. Incubation in a low concentration of sodium hydroxide (NaOH) solution is used for decellularization. A study investigated the effects of NaOH decellularized skin in a porcine and rat full thickness wound model. Optimal incubation time has been shown to be six weeks[28]. Shorter incubation periods (less than four weeks) will not ensure full removal of all the antigenic components, resulting in an inflammatory response. Infiltration of inflammatory cells, such as neutrophils or macrophages (responsible for the production of proteolytic enzymes), leads to a premature degradation of the elastin and collagen matrix. Fibroblasts attach themselves to these ECM components and use them as a lead (scaffold). The fibroblasts will produce new collagen fibres around the donor fibres. This will result in a more favourable random orientation of the fibres and the neo-dermis will have a more natural appearance. Fibroblasts are not able to use prematurely degraded donor-derived fibres as guidance, leading to an undesired parallel (to the epidermis) orientation of newly synthesized collagen fibres. More inflammatory cells, due to a decreased incubation time, might delay wound closure through interference with the outgrowth of the epidermis (from the STSG)[28]. An increase in incubation time (more than eight weeks) can damage the extracellular matrix. The study concluded that decellularization by using a sodium hydroxide solution was not only cost-effective but was also able to preserve the natural elastin and collagen 3D network as well[28].

Preservation of the natural collagen and elastin 3D network is important. In the past, elastin did not get the attention it deserves. Using a dermal substitute with elastin can reduce wound contractures and enhance skin elasticity[41]. Elastin expression is fairly reduced in scar tissue and new elastin fibres are thin, fragmented and less mature than elastic fibres in normal skin. Elastin fibres will never reach the size or maturity of healthy skin, not even after a decade, resulting in hard and inelastic scars. Elastin is not only functionally but also spatially disorganized in scar tissue[19]. It is suggested that the use of dermal substitutes containing both collagen and elastin can increase the deposition of elastin by fibroblasts and replace the destructed elastic fibres[19,41]. This is not the case for dermal substitutes lacking an elastic dermal network, such as Integra®. Glyaderm® contains a native elastic dermal network which has the intact spatial structure of normal human skin, making it the possible next step towards an ideal dermal substitute.

After the incubation time of six weeks, hydrogen chloride (HCl) is added to neutralize the sodium hydroxide. The decellularized skin (Glyaderm®) is then rinsed in a phosphate buffered saline followed by storage in 85% glycerol until needed.

Our first clinical publication of Glyaderm® showed favourable long-term results in 55 patients in a two-stage procedure. We set out to develop a dermal substitute from glycerol preserved allografts more than two decades ago, which was intended to have the following key advantages: native collagen and elastin matrix, easy storage and

handling, inactivation of virus and micro-organisms[42,43] and most importantly, a non-profit product that could be available to a larger number of patients. As clinicians in the field our chief aim was to develop a practical and affordable dermal substitute for burn, cancer and trauma victims.

The most favourable prototype Glyaderm1 was tested in animal studies, which showed favourable results in a three-stage procedure, allograft, Glyaderm1, autograft These promising results prompted the current pilot study and randomized comparison.

There have been many reports attesting the benefits of various dermal substitutes. However, to our knowledge there has been no conclusive randomized trial which demonstrates a superior outcome of skin resurfacing with a dermal substitute and split skin graft over skin resurfacing with a skin graft alone. Most burn experts do not question the value of dermal substitution in surgical burn care and long-term results of patients attest the added value.

Objective scar assessment and longer follow-up is elucidating this advantage, which is already clinically apparent. Our pilot study showed consistent, stable long-term results after 6 years with pliable skin after bi-layered skin restoration with Glyaderm®.

Objective scar assessment showed a significantly improved elasticity of the skin in patients treated with Glyaderm® and skin graft compared to skin graft alone ($p = 0.003$).

Glyaderm® is the first cost-effective, non-commercial, dermal substitute that can be compared with currently available dermal equivalents.

A disadvantage in our initial studies with Glyaderm® was the necessity for three procedures to full wound closure. Direct application of Glyaderm® onto the wound bed without allograft wound bed preparation did not seem to be a viable option in either the animal studies or the phase I pilot study as demonstrated by the 3 patients with a full thickness skin defect after radial forearm flap harvest where, following immediate application of Glyaderm®, we expected no problems in view of the healthy wound bed, but in the end there was no ingrowth of the dermal substitute. The animal studies had also pointed out that simultaneous application of our early Glyaderm® prototype and autograft was not feasible. In Glyaderm® processing a relative dense elastin-collagen network is preserved. Budding capillaries need to penetrate this network before they can nourish the overlying autograft. In addition, the earlier Glyaderm® prototypes were relatively too thick and suffered from batch-to-batch inconsistencies inherent to variation in selection. Continuous research, monitoring of selection and development improved this process of graft selection and standardization.

A purpose designed laser tool is now used to ensure selection of dermis of uniform thickness. The laser accurately scans the distance between the optic and the table and the optic and the Glyaderm® subsequently placed upon the table, allowing the difference in height to be the thickness.

The optimal 0.2-0.4 mm thickness glycerol preserved dermis is now selected for processing into Glyaderm®.

Glyaderm® can be applied with simultaneous skin grafting after wound bed preparation with allografts for 5 days. This improvement has a distinct favourable impact on morbidity and cost[44].

We were surprised to read that De Hennau et al recently reported this simultaneous engraftment from our early clinical study in 2021 as being the first but happy to see that our findings are reproducible by other centres which is our intention[45]. This center that has been using Glyaderm® as a dermal substitute since 2017 found, similar to our results, that this procedure resulted in an excellent average take rate of 98%. In contrast to our protocol, the bilayered skin reconstruction was performed with and without Negative Pressure Wound Therapy (NPWT), resulting both in great results.

Collaboration with researchers from Nijmegen University resulted in visualization of newly synthetized collagen-elastin matrix *in vitro* and *in vivo* with Glyaderm® engraftment.

Finally, we performed "A prospective, controlled, randomized, intra-individual comparative, single-blinded study in a mono-center setting, investigating simultaneous application of Glyaderm®" + split thickness autologous skin grafts (STSG) versus split thickness autologous skin grafts alone in full thickness skin defects and deep burns.

A total of 66 patients were included in this intra-individual study, corresponding to 82 wound comparisons.

The simultaneous application of Glyaderm® and autologous skin proved non-inferior to the previous protocol in terms of graft take, subjective scar scales and scar color. The two-step procedure proved to be superior in terms of elasticity. The visual scar evaluation by the experts one year after wound closure was clearly in favor of Glyaderm® when using the two-step procedure. This was also the case when using the simultaneous application, though not as distinctive as with the two-step procedure. Though we cannot state clear numbers, the costs are undoubtedly to the advantage of the simultaneous application.

Commercially available dermal substitutes are often dealing with reduced tissue vascularization and integration[36]. Budding capillaries experience difficulties penetrating the dermal substitute when they are too dense. Adequate vascularization requires valuable time, thus preventing immediate autografting. Frequently used skin replacements such as Integra® Bilayer and Matriderm® Bilayer have an autografting interval of three weeks[46]. This results in a prolonged inflammatory phase, increasing the possibility of fibrosis and scar retraction. The autografting interval entails an increased infection risk, and this has been proven with Integra® Bilayer[46]. Both Integra® and Matriderm® have developed a 1.00mm single layer product that allows for a single-stage procedure[45]. Glyaderm® has a thickness of 0.30mm and is easily vascularized, enabling immediate autografting. None of the Glyaderm® nor autologous skin was lost due to complications. The skin graft survival was excellent and consistent, indicating the formation of a dermo-epidermic junction. The biopsies

showed adequate vascularization through numerous capillaries. In conclusion, Glyaderm® is easily and adequately vascularized, enabling immediate subsequent autografting.

Many of the biopsies of the Glyaderm®-treated wound sites demonstrated the presence of elastic fibres and the majority of these fibres were organized following a preserved native fiber network pattern. Even in the biopsies that were procured one year after wound closure, donor elastin fibres could be detected. Suggesting that the longevity of the donor elastic fibres is longer than 3 months and likely even surpasses one year. We estimated that the donor collagen would have been cleared away by the time when the first biopsy was procured. Even though it is still an import element in the 3D collagen-elastin network as was stated in the introduction. If the fibroblasts are capable of using this elastic network as a scaffold, it would result in a much more favorable orientation of the scar tissue.

The microbiological analysis of the wound swabs that were harvested throughout the study did not show an increase in bacterial load. This suggests that the risk of infection is not elevated. The objective evaluation of the scar color at long term follow-up has shown that the erythema and pigmentation is not comparable to that of normal skin. The skin of the intervention group shows slight hypopigmentation and increased erythema. The trans epidermal water loss and the skin hydration of the scars treated with Glyaderm® were comparable to those of normal skin. These are desirable characteristics of a functional skin replacement thus concluding that the simultaneous bilayered reconstruction of the skin using Glyaderm® has resulted in the restoration of the skins natural barrier, protecting the patient from danger i.e., hypothermia, infection and dehydration.

Dermal substitutes have been used in the treatment of various medical conditions. Burn injuries however are a special kind of indication. Acute burns often involve large areas resulting in a limited supply of viable autologous donor skin. Additionally, the situation is complicated by intense local and systemic inflammation and there is only a small-time interval for intervention to minimize scarring[48]. However, this was the result of using the two-step procedure. This study is the first large randomized clinical trial to investigate the simultaneous bilayered reconstruction of the skin using Glyaderm®.

This study investigated the applicability of Glyaderm® for burn injuries in an acute setting. Several dermal substitutes such as Integra®, have successfully been used in the reconstruction of chronic burn contractures[43]. Matriderm®, Integra® and Renoskin® can be used for treating patients with exposed bone or tendons[46] In these severe cases simply applying STSG would be insufficient. We also reported on the successful use of Glyaderm® in a burn case complicated by tibial exposure after failure of free flap surgery. Glyaderm®, combined with negative wound pressure therapy and skin grafting could be used as an alternative for flap surgery in selected cases[49].

The variety among dermal substitutes is enormous and different pathways of this healing process are being targeted ensuing different results with every product.

Whether or not the simultaneous application can be improved by using dermal substitution with Glyaderm® with a thickness of over 0.30mm should receive attention in future research.

Quite interestingly, the single most important parameter, the overall opinion of the patient as scored by use of the POSAS, was in favor of Glyaderm® and the gap difference of the two groups increased with every follow-up moment.

Our research group believes that scar quality should be assessed even up to two years after achieving complete wound closure. We believe that defining the final result in scar quality should be at least 1.5 years post wound-closure and preferably even up to 2 years at which time tissue remodeling as well as our standard of care scar therapies are completed.

Conclusion

Within the last two decades we focused on the treatment of full thickness major skin defects by using Glyaderm® as a dermal substitute.

Skin substitutes face unfulfilled challenges such as incapability of providing adequate temperature control or pressure sensation, reduced vascularization due to long-term survival of the replacement, inadequate immune regulation, failed integration, high costs, slow wound healing, infection, pain and unaesthetic scarring[36,50]. The current available cellular skin replacements consist of only two cell types: fibroblasts and keratinocytes. These skin replacements are therefore unable to form specialized structures such as glands or hair follicles[36]. A lot of innovative research has been published during these last few years. A recent study provided the first LGR6+ stem cell-based skin substitute capable of epithelialization, hair growth, and angiogenesis in wound beds[51]. Illustrating a prime example of innovative discovery.

A recent study defined the ideal skin replacement as follows: “An ideal skin substitute, however, would be a durable bilayered construct that is morphologically and biochemically similar to native skin, replicating its texture, structure, and capacity to engraft”[52].

At present, there is no product that can live up to these high-level standards. Glyaderm® however contains a native dermal network which has the intact spatial structure of normal human skin, thus approximating the ideal skin substitute in theory. Distinct advantages of using human derived dermal skin have been stated in the first part of this dissertation. The use of human derived dermal substitutes has drawbacks i.e., limited supply of donor skin, possible ethical problems, slower endothelial cell penetration and the lack of skin appendages[46]. Research has been ongoing for many years and important progress has been made. Technologies that were once considered “the future” are making their entry. Electrospinning, recombinant proteins, small-molecule engineering, stem cells autologous cultured dermal substitutes and three-dimensional bio-printing are just a few examples of the modern approach in burn care[48,50].

As mentioned before, burn injuries contribute considerably to the mortality and morbidity of the population worldwide. In 2004, nearly 11 million people were severely burned and required medical treatment[50]. Post-trauma burn-victims are left with cosmetic disfigurement, impaired functions, psychological trauma, problems with activities of daily life and social dysfunction[53]. The goal of burn care is, as it has always been, not merely the reconstruction of the damaged tissue, but rather the complete restoration of the patient as a whole. Only one of many possible solutions to achieve this is the use of a dermal substitute. The results of this study have provided us with interesting data. Not only did we find that the simultaneous application of Glyaderm® and STSG was possible, but that the donor elastin fibres were histologically detectable even one year after achieving complete wound closure. The bilayered reconstruction using Glyaderm® was non-inferior to both the gold standard and the two-step procedure in many ways. Additionally, the tissue that was reconstructed with Glyaderm® had numerous features that resembled those of healthy human skin. However, the search for a world-wide available, easy-applicable and cost-effective solution remains.

We have intended to demonstrate the scope of application of Glyaderm® as a DRM for plastic surgeons and burn surgeons.

Glyaderm® has been successfully used for indications other than deep burns and burn scars i.e., oncological resections, free flap donor site reconstructions, giant melanocytic nevi and post necrotizing fasciitis reconstructions[46].

We remain committed to our initial goal and intention to make Glyaderm® DRM available for widespread application in burns. To this end strong collaboration with plastic surgeons in Colombia has resulted in Glyaderm® being successfully produced at the Bogota Skin Bank and applied in one stage and two procedures in major (facial) burns. Also, royalties from book and charitable organizations are procuring funding to make Glyaderm® available for patients with major burn and traumatic full thickness defects.

A gamut of research must be done until the perfect off-the-shelf skin replacement and acellular matrix becomes available.

Glyaderm® is an optimal non-commercial dermal regeneration matrix and can serve to bridge this gap in the near future and also be a conduit matrix for further tissue-engineering studies[54].

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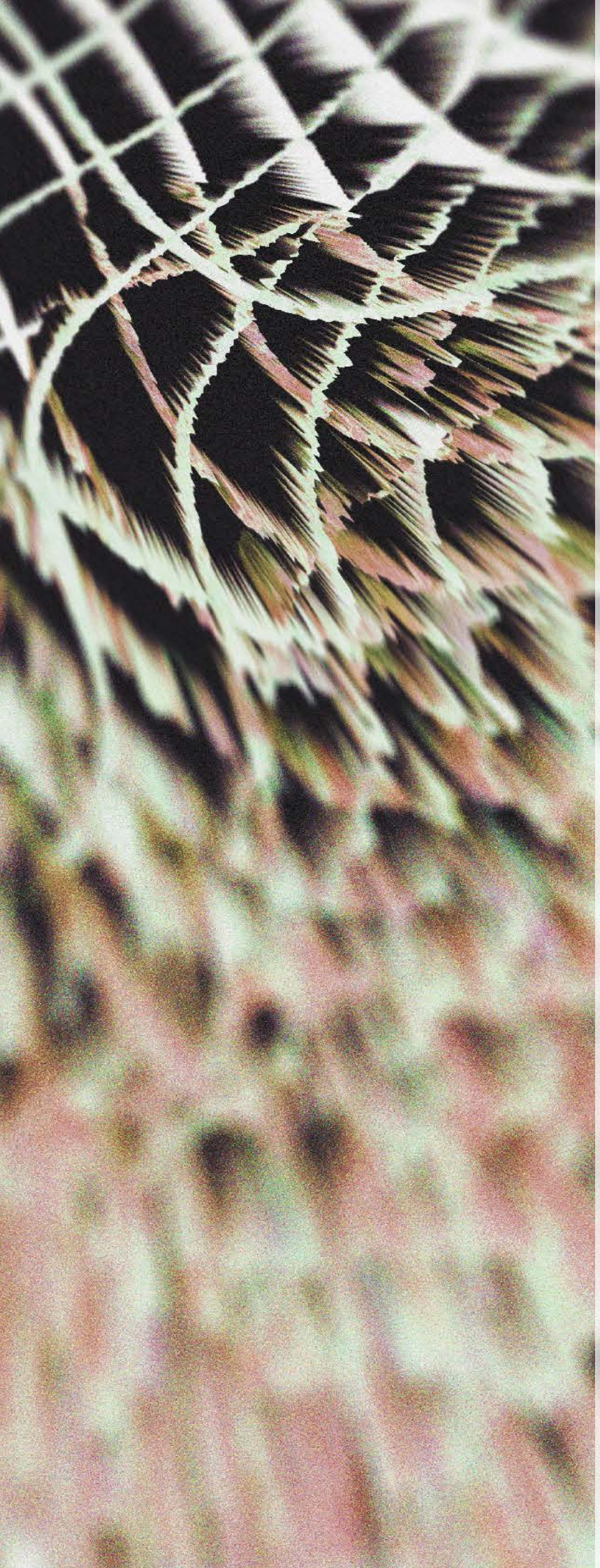

CHAPTER 12

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

General discussion and future perspectives.

Burns represent a significant cause of trauma-related mortality, with varying incidence rates globally. Recent advancements in burn care have improved patient survival and recovery, leading to a shift in focus from mere survival to enhancing the patient's quality of life.

Quality of life for burn patients is dependent on several factors, including reintegration into society, scar appearance and quality, and self-perception of appearance. Autologous split-thickness skin graft (STSG) is the current gold standard treatment for deep dermal and full-thickness burns. However, STSG is associated with several challenges, including limited donor site availability, donor site morbidity, contracture, and unpredictable scar healing. In addition to human allografts, biological and synthetic skin substitutes have been developed for burn treatment.

Dermal regenerative matrices (DRMs) are permanent skin substitutes that aid dermal skin component regeneration in the treatment of total skin defects resulting from burns, traumatic wounds, or burn contracture resolution. DRMs have demonstrated beneficial functional and aesthetic outcomes in both acute burn surgery and burn reconstruction. Nonetheless, DRMs have drawbacks, including the need for a two-stage procedure, increased infection risk, and high cost.

Historical aspects

At the turn of the millennium, our research group faced challenges in the reconstruction of burn care. The DRM Integra received increasing attention in the literature and at conferences, but the variability in “take” or ingrowth and the very high cost prevented widespread application and routine use in the Ghent Burn Unit. Having witnessed brutal burn & trauma of revolution and war as a child in Iran, Pirayesh was intrigued by Plastic Surgery & Burn Care as a Senior House Officer in the East Grinstead Burn Unit, where his mentor Philip Gilbert taught him the principles of burn care. The Queen Victoria Hospital was famous since the Second World War for Sir Archibald McIndoe who bravely treated the burns of RAF pilots from the Battle for Britain. He started researching keratinocyte culture and presented papers at burns conferences where he met Hans Hoekstra, the inventor of the glycerol conserved allograft (GPA). Hoekstra was active in experimental burns research in Amsterdam and taught Pirayesh the core principles of experimental burns research together with Dr. Nelleke Richters who worked as an immunologist and researcher for the Dutch Burns Foundation.

Pirayesh was impressed by the research output of the Ghent Plastic Surgery Department and approached Prof. Stan Monstrey at a conference which gave him the opportunity to apply for a training position. Pirayesh was selected for training as a plastic surgeon but had to start with a pre-residency year at the Ghent Burns Unit.

Henk Hoeksema, chief burn care coordinator, taught him the principles of conservative burn care and surgical burn care. They introduced and started studies with MEEK transplantation and interactive honey dressings in the Ghent Burn Unit, which was known for using laser-doppler imaging to scientifically delineate the depth of burns and was therefore the ideal unit for clinical studies on burns. Their joint brainstorming sessions culminated in the idea of developing a dermal substitute based on glycerol-preserved allograft, ideally on a non-commercial basis and cost-effective DRM for wide application and improvement of the quality of life of burn patients.

Pirayesh, Hoeksema, Richters, Hoekstra and Monstrey abdicated their IP rights for the DRM "Glyaderm", which was notarized at the EuroSkinBank, now EuroTissueBank, Beverwijk, The Netherlands.

Pirayesh returned to the Netherlands to build his own practice, but propagated Glyaderm® research and worldwide application, resulting in the development of a Colombian Glyaderm® for acid attack victims. The charity Two Faces (https://twofacesfoundation.org) was founded to help these victims by his wife Eva Velders. Berend van der Lei has been an inspirational force throughout his career and has coached him to structure and submit this thesis under his supervision together with Prof. Monstrey now that the long-term results of the studies are available that will reinforce the position of Glyaderm® among other dermal regeneration matrices.

Glyaderm®

Glyaderm® is the result of processing glycerol-preserved allogeneic donor skin. Using glycerol has some significant benefits. Skin preservation with glycerol is not only more cost-effective than cryopreservation, GPA is also less immunogenic. Glycerol has bactericidal properties; 97% of GPA bacteriological cultures are negative after 3 months. Glycerol can inactivate viruses such as HIV-1 and Herpes Simplex. The donor cells are not viable due to the glycerol preservation method, but the collagen and elastin network remain intact. With production of Glyaderm®, all donor cells (i.e., hair cells, vascular endothelium, smooth muscle, and keratinocytes) must be removed to prevent an adverse inflammatory (immunogenic) response leading to neodermal rejection. The main advantage of using a GPA-derived dermal substitute is that it resembles the natural collagen-elastin structure of human skin. This in contrast to other dermal substitutes, such as Matriderm® or Integra®, which are of animal and/or synthetic origin. It is preferable to maintain the natural collagen and elastin 3D fiber network of the dermis. A few years ago, a panel of experts stated: *"Given current knowledge, the ideal acellular matrix is one that most closely approximates the structure and function of the human extracellular matrix (ECM) it replaces".* The glycerol remnants are removed by thoroughly rinsing the GPA in sterile saline (NaCl). Repeated washing is done to ensure residual glycerol is removed. Incubation in low concentration sodium hydroxide (NaOH) solution is the method used for decellularization. We investigated the effects of NaOH-decellularized skin in a pig and rat full-thickness wound model. The optimal incubation period has been found to be six weeks. Shorter incubation periods (less

than four weeks) do not guarantee complete removal of all antigenic components, resulting in an inflammatory response. Infiltration of inflammatory cells, such as neutrophils or macrophages (responsible for the production of proteolytic enzymes), leads to a premature breakdown of the elastin and collagen matrix. Fibroblasts attach to these ECM components and use them as a scaffold. The fibroblasts start to produce new collagen fibers around the donor fibers. This will result in a more favorable random orientation of the fibers and the neodermis will appear more natural. Fibroblasts are unable to use the prematurely degraded donor-derived fibers as a guide, leading to an undesirable parallel (to the epidermis) orientation of newly synthesized collagen fibers. More inflammatory cells, due to a shorter incubation time, could delay wound closure by interfering with the outgrowth of the epidermis (of the STSG). An extension of the incubation period (more than eight weeks) may damage the extracellular matrix.

Our study showed that decellularization by using a sodium hydroxide solution was not only cost-effective, but also able to preserve the natural elastin and collagen 3D network. Preservation of the natural collagen and elastin 3D network is important. In the past, elastin didn't get the attention it deserves. Using a dermal substitute containing elastin can reduce wound contractures and improve skin elasticity. Elastin expression is quite reduced in scar tissue and new elastin fibers are thin, fragmented and less mature than elastic fibers in normal skin. Elastin fibers will never reach the thickness or maturation level of healthy skin, even after ten years, resulting in hard and inelastic scars. Elastin is not only functional but also spatially disorganized in scar tissue. It is suggested that the use of dermal substitutes containing both collagen and elastin may increase elastin production by fibroblasts and replace lost elastic fibers. This is not the case for dermal substitutes that do not have a human elastin network, such as Integra®. Glyaderm® contains a human elastin dermal network with the intact spatial structure of normal human skin, potentially making it a step further towards developing an ideal dermal substitute.

After the six-week incubation period, hydrogen chloride (HCl) is added to neutralize the sodium hydroxide. The decellularized skin (Glyaderm®) is then rinsed in phosphate buffered saline, after which it can be stored in 85% glycerol until ready for use. No special storage space is required.

Studies to develop and validate Glyaderm®

Different incubation periods in NaOH were used to prepare dermal matrix prototypes from donor skin, ranging from 2 to 8 weeks.

Standard histology techniques were employed to analyze the resulting prototypes, which were subsequently tested in both rat and pig models. In the rat model, all prototypes exhibited intact biocompatibility four weeks after implantation, as evidenced by the presence of ingrown blood vessels and fibroblasts. However, an inflammatory response was observed in prototypes treated with NaOH for only 2 or 4 weeks. In the pig model, the prototypes treated with 6 or 8 weeks of NaOH were able

to reduce wound contraction. An optimal incubation period of 6 weeks was determined, as longer periods caused damage to the collagen fibers.

The elastin fibers were well preserved in all prototypes. In the neodermis of pig wounds treated with 6 or 8 weeks of NaOH, elastin fibers originating from the prototype were observed 8 weeks after surgery, surrounded by more randomly oriented collagen fibers. The results suggest that an effective dermal matrix can be obtained from glycerol-preserved donor skin.

Further clinical studies are planned to assess the potential of this material for dermal substitution in deep burn wounds (**Chapter 2).**

In our study, we compared an acellular dermal substitute (Glyaderm®) prepared from glycerol-preserved human skin with already known substitutes, using a pig wound model. The donor cells were removed by incubation in 0.06 M NaOH solution, and the substitutes were applied to full-thickness wounds covered with an STSG. A two-stage procedure was used for Glyaderm®, with the STSG placed a week after application. The response to wound healing was analyzed macroscopically and on biopsies over 8 weeks, and the survival of the STSG was compared to control wounds.

In the first series of experiments, the inflammatory response and influx of myofibroblasts in Glyaderm® were limited, indicating possible beneficial outcomes on final wound healing outcomes. However, the survival of the STSG on the acellular dermis was lower compared to control wounds. In the second series, the "take" of the STSG was the same as controls, but the wound contraction was reduced. The application of Glyaderm® was not inferior to Integra® in reducing wound contraction when applied in a two-stage procedure.

In conclusion, our study suggests that Glyaderm® can be successfully used in a two-stage procedure to reduce wound contraction. Further studies are needed to evaluate its efficacy in other wound types and in clinical settings **(Chapter 3).**

In this subsequent study, we evaluated the integrity and biocompatibility of our "Glyaderm" dermal replacement matrix (DRM) in repairing abdominal wall defects. Abdominal wall repair can be performed using synthetic or biological matrices, with biological materials potentially reducing the risk of infections and fibrosis. The study aimed to compare two acellular human dermis products, with one being prepared using low concentrations of NaOH (i.e., Glyaderm®) and the other being SureDerm®, a commercially available dermal substitute. We used a rat model to compare the two materials, in which full-thickness defects were closed with the matrices. The rats were sacrificed 1 or 4 months after surgery, and the number of intestinal adhesions was noted. Histological analysis and measurement of tensile strength were also performed on tissue samples.

Both groups showed good functional integration of the implants with the abdominal wall. The group treated with the NaOH prototype (Glyaderm®) showed no intestinal adhesion, whereas 4 out of 7 rats in the SureDerm® group showed only minor adhesions after 4 months. The tensile strength of the healed tissue was significantly higher in the NaOH prototype group at 4 months after surgery ($p < 0.0026$). These

results suggest that both human acellular dermis products can be used in clinical trials for the closure of abdominal wall defects **(Chapter 4).**

We then performed literature review on skin replacement for burns. The goal of this study was to provide an overview of which types of skin substitutes have been developed and which questions still need to be answered. None of the commercialized products can currently claim to be the optimal dermal substitute, mainly because clinical evidence is too scarce. The number of products being commercialized is nevertheless steadily increasing, which calls for a certain overview, classification, and clear comparison of the available products **(Chapter 5).**

Adverse post-burn scarring is a significant problem that affects a large number of individuals. Consequently, a majority of scar assessment and treatment studies have focused on burn scars due to their relatively high prevalence. While surgical and dermatological scars may also result in scarring, their impact is usually more limited, and thus, they are less well studied. Therefore, burn scars are likely the scars with the most significant impact on quality of life. Excessive scarring can lead to both physical and psychological effects that can impede an individual's quality of life, including painful and lengthy treatments that may yield suboptimal outcomes. Scars can also cause discomfort, itching, and pain, and contractures can limit mobility and function. The integration of individuals with hypertrophic scars in a society where physical appearance has become increasingly important can also pose challenges. Burn scars can have a considerable psychological impact on affected individuals, as they are highly visible and stigmatizing, similar to other severe chronic dermatological conditions.

Despite the importance of scar assessment, it remains a neglected area, and there is still no consensus on the ideal scar assessment method, despite the many scales and tools that have been developed over the past few decades. However, adequate scar assessment is crucial for clinical evaluation and follow-up, and it is also essential to compare different wound or scar treatment modalities. In addition, an objective scar evaluation may be required for medico-legal reasons, such as reimbursement of treatments and proof of disability.

Scar evaluation can be performed using simple paper and pencil scar scales that rate several variables, often through subjective word descriptions (such as red or raised). However, more technically sophisticated and objective devices, such as spectrometry or ultrasound, can analyze one or more variables in a more reproducible manner. The aim of our research was to provide an analysis and critical overview of the scar scales developed to assess the aesthetic and physical aspects of burn scars and their role in burn assessment.

To achieve this, we investigated the available scar tools that can be used in burn assessment and scar research. Unfortunately, there is a paucity of literature on scar tools available for scar assessment. Therefore, our research focused on identifying the available scar scales that can be used to assess burn scars. We analyzed the various scales and tools that have been developed to assess burn scars, focusing on their advantages, disadvantages, and validity. We also reviewed the evidence on the

correlation between scar assessment scores and clinical outcomes, such as pain, itching, and mobility.

Our study has several implications for scar assessment and research. Firstly, our findings underscore the importance of using a standardized and objective approach to scar assessment to improve the comparability and reliability of results. Secondly, our study highlights the need for further research into the development and validation of scar scales and tools. Finally, our study emphasizes the need for scar assessment to be an integral part of burn assessment and treatment to optimize clinical outcomes and improve patients' quality of life **(Chapters 6 and 7)**.

The development of an effective and affordable skin substitute for burn, cancer, and trauma victims has been a long-standing goal of medical researchers. In this regard, glycerol-preserved allografts have been identified as a promising material for developing a dermal substitute due to their human collagen and elastin matrix, ease of storage and handling, inactivation of viruses and microorganisms, and non-profit availability. The most favorable prototype of this substitute, Glyaderm®, has been tested in animal studies and a pilot study on humans. This paper aims to provide an overview of the results of the first clinical publication of Glyaderm® and discuss the key findings of the study.

The study was conducted on 55 patients who underwent a two-stage procedure for skin restoration with Glyaderm®. The pilot study involved wound bed preparation with allografts for five days followed by simultaneous application of Glyaderm® and autograft for wound closure. Objective scar assessment was performed at regular intervals up to six years post-treatment. The study compared the outcomes of patients treated with Glyaderm® and skin graft with those treated with skin graft alone.

The study demonstrated that Glyaderm® is a cost-effective and non-commercial dermal replacement that is comparable to currently available dermal equivalents. The long-term results of the study showed consistent and stable outcomes, with patients exhibiting supple skin after six years of treatment. Objective scar assessment showed that patients treated with Glyaderm® and skin graft had significantly improved skin elasticity compared to those treated with skin graft alone ($p = 0.003$). The study also highlighted the benefits of dermal replacement in surgical burn care and its added value in long-term patient outcomes.

The study's findings are significant in that they demonstrate the effectiveness of Glyaderm® as a viable dermal substitute. The study's results confirm the earlier promising results seen in animal studies and the pilot study. The use of allografts for wound bed preparation was found to be necessary for successful application of Glyaderm®. Direct application of Glyaderm® to the wound bed without wound bed preparation was not a viable option. The study also identified the optimal thickness of glycerol-preserved dermis for processing into Glyaderm® (0.2-0.4mm). The study showed that simultaneous application of Glyaderm® and autograft after wound bed preparation with allografts was an effective procedure for wound closure, reducing morbidity and costs.

The first clinical publication of Glyaderm® demonstrated its favorable long-term results in 55 patients in a two-stage procedure. The study confirms the effectiveness

of Glyaderm® as a cost-effective and non-commercial dermal replacement that can be compared to currently available dermal equivalents. The study also highlighted the benefits of dermal replacement in surgical burn care and long-term patient outcomes. The study's findings have advanced our understanding of the use of glycerol-preserved allografts for developing a dermal substitute and identified the optimal thickness of glycerol-preserved dermis for processing into Glyaderm®. The study's findings will be useful for clinicians in the field in developing practical and affordable skin replacements for victims of burns, cancer, and trauma **(Chapter 8).**

We were surprised to read that De Hennau et al recently (2021) reported this simultaneous transplant reported in our early clinical trial as “the first”, but pleased to see that our findings are reproducible by other centers, which is also our intention. This center, which has been using Glyaderm® as a DRM since 2017, found, similar to our results, that this procedure resulted in an excellent average absorption rate of 98%. In contrast to our protocol, the bilayer skin reconstruction was performed with and without Negative Pressure Wound Therapy (NPWT), both of which resulted in favorable results.

Collaboration with researchers from the University of Nijmegen resulted in visualization with histochemical techniques of newly synthesized collagen-elastin matrix *in vitro* and *in vivo* with Glyaderm® implantation reported in Scientific Reports (Nature) **(Chapter 9).**

Finally, we conducted "A prospective, controlled, randomized, intra-subject comparative, single-blind study in a monocentric setting, investigating the concomitant application of Glyaderm®" + autologous skin grafts (STSG) versus autologous skin grafts (STSG) alone in complete thickness skin defects full and deep burns.

A total of 66 patients were included in this intra-individual study, corresponding to 82 wound comparisons.

The simultaneous application of Glyaderm® and autologous skin proved non-inferior to the previous protocol in terms of graft uptake, subjective scar scales and scar color. The two-step procedure proved to be superior in terms of elasticity. The experts' visual scar evaluation one year after wound closure clearly favored Glyaderm® using the two-step procedure. This was also the case when using the simultaneous application, but not as distinctly as with the two-step procedure. Although we cannot give unequivocal figures, the costs are undoubtedly in favor of the simultaneous application of Glyaderm® with STSG in 1 operation.

Commercially available dermal substitutes often suffer from reduced tissue vascularization and integration. Budding capillaries have difficulty penetrating the DRMK when they are too dense. Adequate vascularization requires valuable time, preventing immediate autotransplantation. Commonly used DRMs such as Integra® Bilayer and Matriderm® Bilayer have an autotransplant interval of three weeks. This results in a prolonged inflammatory phase, increasing the risk of fibrosis and scar retraction. The autotransplant interval carries an increased risk of infection, and this has been proven with Integra® Bilayer. Both Integra® and Matriderm® have developed a single-layer 1.00 mm product that allows for a one-stage procedure.

Glyaderm® has a unique human collagen-elastin matrix with a thickness of 0.30 mm and is easily vascularized, allowing immediate autotransplantation. None of the Glyaderm® or autologous skin was lost due to complications. Skin graft survival was excellent and consistent, indicating the formation of a dermoepidermic junction. The biopsies showed adequate vascularization through numerous capillaries. In conclusion, Glyaderm® is easily and adequately vascularized, allowing simultaneous STSG autotransplantation.

Many of the biopsies of the wound sites treated with Glyaderm® showed the presence of elastic fibers and most of these fibers were organized according to a preserved network pattern of natural fibers. Even in the biopsies taken one year after wound closure, donor elastin fibers could be detected. This suggests that the lifespan of the donor elastic fibers is longer than 3 months and probably even longer than a year. We estimate that the donor collagen would have been removed by the time the first biopsy was obtained. Even though it is still an important element in the 3D collagen-elastin network, as stated in the introduction. If the fibroblasts can use this elastic network as a matrix, this would result in a much more favorable orientation of the scar tissue.

The microbiological analysis of the wound swabs taken during the study showed no increase in bacterial load. This suggests that the risk of infection is not increased. The objective evaluation of the scar color at long-term follow-up has shown that erythema and pigmentation are not comparable to those of normal skin. The skin of the intervention group shows slight hypopigmentation and increased erythema. The transepidermal water loss and skin hydration of the Glyaderm® treated scars were comparable to normal skin. These are desirable features of a functional skin replacement, from which it can be concluded that the simultaneous bilayer reconstruction of the skin using Glyaderm® has resulted in the restoration of the skin's natural barrier, protecting the patient from danger i.e., hypothermia, infection, and dehydration.

Skin substitutes have been used in the treatment of a variety of medical conditions. However, burns are a special kind of indication. Acute burns often involve large areas, resulting in a limited supply of viable autologous donor skin. In addition, the situation is complicated by intense local and systemic inflammation and there is only a small window for intervention to minimize scar formation. However, this was the result of using the two-step procedure. This study is the first large randomized clinical trial to investigate simultaneous bilayer reconstruction of the skin using Glyaderm®.

This study investigated the applicability of Glyaderm® in burns in an acute setting. Several dermal substitutes, such as Integra®, have been successfully used in the reconstruction of chronic burn contractures. Matriderm®, Integra® and Renoskin® can be used to treat patients with exposed bone or tendons. In these severe cases, applying STSG is insufficient. We also reported on the successful use of Glyaderm® in a case of burns complicated by tibial bone exposure following the failure of free flap surgery. In selected cases, Glyaderm®, combined with negative wound pressure therapy and skin grafting, can be used as an alternative to lap surgery.

The variety of dermal substitutes is huge, and research is done in different ways, resulting in different results with each product. Whether the simultaneous application can be improved by using dermal substitution with Glyaderm® with a thickness greater than 0.30 mm needs to be addressed in future research.

Very interestingly, the single most important parameter, the patient's overall experience and feeling as scored by using the POSAS, was in favor of Glyaderm® and the difference in favor of Glyaderm® group increased with each follow-up time.

Our research group believes that scar quality should be assessed even up to two years after complete wound closure. We believe that the final result in scar quality should be at least 1.5 years after wound closure and preferably even up to 2 years when tissue remodeling and our standard scar therapy treatments are complete **(Chapter 10).**

Conclusion

Over the past two decades, we have focused on the treatment of major skin defects by using Glyaderm® as a dermal substitute.

Dermal substitutes face particular challenges, such as the inability to provide adequate temperature control or pressure sensation, reduced vascularization due to prolonged survival of the substitute, inadequate immune regulation, failed integration, high cost, slow wound healing, infection, pain, and unaesthetic scarring. Currently available cellular skin substitutes consist of only two cell types: fibroblasts and keratinocytes. These skin replacements are therefore unable to form specialized structures such as glands or hair follicles. A gammut of innovative research has been published in recent years. A recent study yielded the first LGR6+ stem cell-based skin substitute capable of epithelization, hair growth and angiogenesis in wound beds. To illustrate a good example of innovative discovery.

Another recent study defined the ideal skin replacement as follows: “However, an ideal skin replacement would be a durable bilayer reconstruction that is morphologically and biochemically similar to the original skin, mimicking its texture, structure and ability to regenerate".

At present day there is no product that can meet all these high requirements. However, Glyaderm® contains a natural dermal network that has the intact spatial structure of normal human skin, making it the closest theoretical approach to the ideal skin substitute. In the first part of this thesis, the clear advantages of using donor skin of human origin have been mentioned. The use of human-derived dermal substitutes has drawbacks such as a limited supply of donor skin, potential ethical issues, slower penetration of endothelial cells, and the lack of skin appendages. Research has been going on for many years and significant progress has been made. Technologies once considered "the future" are making their appearance. Electrospinning, recombinant proteins, small molecule engineering, autologous cultured skin substitutes using stem cells and three-dimensional bioprinting are just a few examples of the modern approach in burn care not to mention the promise of artificial intelligence.

As mentioned earlier, burn injuries contribute significantly to the mortality and morbidity of the population worldwide. In 2004, nearly 11 million people were severely burned and required clinical medical treatment. Post-trauma burn victims are left with cosmetic deformities, impaired functions, psychological trauma, difficulty with daily activities, and social dysfunction. The goal of burn care, as always, is not just the reconstruction of the damaged tissue, but the full recovery of the patient as a whole. Just one of many possible solutions to achieve this is to use a dermal substitute. The results of our studies have provided us with interesting data. Not only did we find that the simultaneous application of Glyaderm® and STSG was possible, but that the donor's elastin fibers were histologically detectable even one year after the wound had completely closed. The double-layer reconstruction with Glyaderm® was in many ways equal to both the gold standard and the two-step procedure. In addition, the tissue reconstructed with Glyaderm® had many features similar to those of healthy human skin.

It is our intention to educate and propagate the application scope of Glyaderm® as a DRM for plastic surgeons and burn surgeons.

Glyaderm® has been successfully used for indications other than deep burns and burn scars, i.e., oncologic resections, free flap donor site reconstructions, giant melanocytic naevi and reconstructions of post-necrotizing fasciitis.

We remain committed to our original goal and intent to make Glyaderm® DRM available for widespread application in burns. To this end, a strong collaboration with plastic surgeons in Colombia has led to Glyaderm® being successfully produced at the Bogota Skin Bank and applied in one stage and two procedures for severe (facial) burns. Also, royalties from book and charitable organizations provide funding to make Glyaderm® available to patients with severe burns and full-thickness traumatic defects.

A plethora of research needs to be done now and in the future until the perfect ready-to-use skin replacement and acellular matrix becomes available.

Glyaderm® can be a viable DRM to bridge this gap to improve the quality of life of many victims of trauma and burns now and in the near future. In addition, Glyaderm® can serve as a biological dermal matrix for further cell regeneration and tissue engineering research in the quest for continuing tissue regeneration.

CHAPTER 13

NEDERLANDSE SAMENVATTING (DUTCH SUMMARY)

ACKNOWLEDGEMENTS & CURRICULUM VITAE

Introductie

Brandwonden vormen een van de belangrijkste doodsoorzaken van trauma gerelateerde sterfte en de incidentie varieert wereldwijd.

Aanzienlijke vorderingen in de zorg voor brandwonden die de afgelopen decennia zijn gemaakt, hebben geleid tot een verbeterde overleving en herstel van de patiënt, waardoor het primaire doel van de behandeling van ernstige brandwonden is verschoven van louter overleven naar het verbeteren van de "kwaliteit" van het leven van de patiënt.

Kwaliteit van leven hangt grotendeels af van hoe patiënten re-integreren in de samenleving, de littekenkwaliteit en het uiterlijke aspect ervan, en de perceptie van hun eigen uiterlijk.

Autologe split-thickness skin graft (STSG) is de huidige gouden standaard voor de behandeling van diepe dermale en volledige dikte brandwonden, maar er zijn tal van uitdagingen verbonden aan STSG's, waaronder beperkte beschikbaarheid van de donorplaats, morbiditeit op de donorplaats, contractuur en een onvoorspelbaar of soms slecht littekengenezing.

Naast humane allotransplantaten zijn er de laatste decennia epidermale en/of dermale biologische en synthetische huidvervangers op de markt gekomen.

Dermale regeneratieve matrices (DRM's) zijn permanente huidvervangers die worden gebruikt om regeneratie van de dermale huidcomponent mogelijk te maken bij het behandelen van totale huiddefecten die zijn achtergebleven na excisie van diepe brandwonden, traumatische wonden of na het opheffen van brandwondcontracturen.

DRM heeft ook toepassing bij acute brandwondenchirurgie en reconstructie van brandwonden en het is aangetoond dat het voor beide indicaties gunstige functionele en esthetische resultaten oplevert.

Naast de vele voordelen, zou DRM ook verschillende nadelen hebben, waaronder de noodzaak van een procedure in twee fasen, een verhoogd infectierisico en hoge kosten.

Historische aspecten

Aan het begin van het millennium stond onze onderzoeksgroep voor uitdagingen in de reconstructie van de brandwondenzorg. De DRM Integra kreeg toenemend aandacht in de literatuur en op conferenties, maar de variabiliteit in “take” of ingroei en de zeer hoge kosten verhinderden een wijdverbreide toepassing en routinematig gebruik in de Gentse Brandwondenafdeling. Pirayesh, die als kind in Iran getuige was geweest van het wrede brandwondentrauma in revolutie en oorlog, werd door Plastic Surgery & Burn Care gegrepen als Senior House Officer op de brandwondenafdeling in East Grinstead, waar zijn mentor Philip Gilbert hem de principes van brandwondenzorg leerde. De Queen Victoria Hospital was befaamd sinds de tweede wereldoorlog door Sir Archibald McIndoe die dapper de brandwonden van RAF-piloten van de Battle for Britain had behandeld. Hij begon onderzoek naar

keratinocytenkweek en presenteerde papers op brandwondenconferenties waar hij Hans Hoekstra ontmoette, de uitvinder van de glycerol geconserveerde allograft (GPA). Hoekstra was actief in experimenteel brandwondenonderzoek in Amsterdam en leerde Pirayesh de kernprincipes van experimenteel brandwondenonderzoek samen met dr. Nelleke Richters die als immunoloog en onderzoeker werkte voor de Nederlandse Brandwonden Stichting.

Pirayesh was onder de indruk van de onderzoeksoutput van de Gentse Plastische Chirurgische Afdeling en benaderde prof. Stan Monstrey op een conferentie die hem de kans gaf om een opleidingsplaats aan te vragen. Pirayesh werd geselecteerd voor de opleiding tot plastisch chirurg maar moest beginnen met een pre-residentiejaar op de Gentse Brandwondenafdeling. Henk Hoeksema, hoofdcoördinator brandwondenzorg, leerde hem de beginselen van conservatieve brandwondenzorg en chirurgische brandwondenzorg. Ze introduceerden en startten studies met MEEK-transplantatie en interactieve honingverbanden in de afdeling Gent, die bekend stond om het gebruik van laser-doppler-beeldvorming om de diepte van brandwonden wetenschappelijk af te bakenen en daarom de ideale plaats was voor klinische studies over brandwonden. Hun gezamenlijke brainstormsessies mondden uit in het idee om een dermaal substituut te ontwikkelen op basis van glycerol geconserveerde allotransplantaat, idealiter op niet-commerciële basis en kosteneffectief DRM voor brede toepassing en verbetering van de kwaliteit van leven van patiënten met brandwonden.

Pirayesh, Hoeksema, Richters, Hoekstra en Monstrey namen afstand van hun IP-rechten voor de DRM "Glyaderm", die door notaris werd bekrachtigd bij de EuroSkinBank, nu EuroTissueBank, Beverwijk, Nederland.

Pirayesh keerde terug naar Nederland om zijn eigen praktijk op te bouwen, maar propageerde Glyaderm®-onderzoek en wereldwijde toepassing, wat resulteerde in de ontwikkeling van een Colombiaanse Glyaderm® voor slachtoffers van zuuraanvallen. De liefdadigheidsinstelling Two Faces (https://twofacesfoundation.org) is opgericht om deze slachtoffers te helpen door zijn vrouw Eva Velders. Berend van der Lei is gedurende zijn hele carrière een inspirerende kracht geweest en heeft hem gecoacht om dit proefschrift onder zijn leiding samen met prof. Monstrey te structureren en in te dienen nu de langetermijnresultaten van de onderzoeken beschikbaar zijn die de plaats van Glyaderm® onder andere dermale regeneratiematrices bevestigen.

Glyaderm®

Glyaderm® is het resultaat van de verwerking van met glycerol geconserveerde allogene donorhuid. Het gebruik van glycerol heeft enkele significante voordelen. Huidconservatie met glycerol is niet alleen goedkoper dan cryopreservatie, GPA is ook minder immunogeen. Glycerol heeft bacteriedodende eigenschappen; 97% van de bacteriologische kweken van GPA is na 3 maanden negatief. Glycerol kan virussen zoals HIV-1 en Herpes Simplex inactiveren. De donorcellen zijn niet levensvatbaar door de glycerolconserveringsmethode, maar de collageen- en elastinenetwerken blijven intact. Bij Glyaderm® moeten alle donorcellen (dwz

haarcellen, vasculair endotheel, gladde spieren enkeratinocyten) worden verwijderd om een ongunstige inflammatoire (immunogene) respons te voorkomen die leidt tot afstoting van de neodermis. Het belangrijkste voordeel van het gebruik van een van GPA afgeleide dermale vervanger is dat het lijkt op de natuurlijke collageen-elastine structuur van de menselijke huid. Dit in tegenstelling tot andere dermale substituten, zoals Matriderm® of Integra® die van dierlijke en/of synthetische oorsprong zijn. Het essentieel om het natuurlijke collageen- en elastine 3D-vezelnetwerk van de dermis te behouden. Een paar jaar geleden verklaarde een panel van experts: "Gezien de huidige kennis is de ideale acellulaire matrix er een die de structuur en functie van de humane extracellulaire matrix (ECM) die het vervangt het dichtst benadert" 30. De glycerol wordt verwijderd door de GPA in een steriele zoutoplossing (NaCl) grondig te spoelen. Er wordt herhaaldelijk gewassen om ervoor te zorgen dat resterende glycerol wordt verwijderd. Incubatie in een lage concentratie natriumhydroxide (NaOH)-oplossing is de gebruikte methode voor decellularisatie. We onderzochten de effecten van met NaOH van cellen ontdane huid in een wondmodel van volledige dikte van varkens en ratten. De optimale incubatietijd is zes weken gebleken. Kortere incubatieperioden (minder dan vier weken) garanderen niet de volledige verwijdering van alle antigene componenten, wat resulteert in een ontstekingsreactie. Infiltratie van ontstekingscellen, zoals neutrofielen of macrofagen (verantwoordelijk voor de productie van proteolytische enzymen), leidt tot een voortijdige afbraak van de elastine- en collageenmatrix. Fibroblasten hechten zich aan deze ECM-componenten en gebruiken ze als leidraad (scaffold). De fibroblasten gaan nieuwe collageenvezels produceren rond de donorvezels. Dit zal resulteren in een gunstiger willekeurige oriëntatie van de vezels en de neodermis zal er natuurlijker uitzien. Fibroblasten zijn niet in staat de voortijdig afgebroken, van donor afkomstige vezels als leidraad te gebruiken, wat leidt tot een ongewenste parallelle (ten opzichte van de epidermis) oriëntatie van nieuw gesynthetiseerde collageenvezels. Meer ontstekingscellen, als gevolg van een kortere incubatietijd, zouden de wondsluiting kunnen vertragen door interferentie met de uitgroei van de opperhuid (van de STSG). Een verlenging van de incubatietijd (meer dan acht weken) kan de extracellulaire matrix beschadigen.

Onze studie toonde aan dat decellularisatie door een natriumhydroxide-oplossing te gebruiken niet alleen kosteneffectief was, maar ook in staat was om het natuurlijke elastine- en collageen 3D-netwerk te behouden. Behoud van het natuurlijke collageen en elastine 3D-netwerk is belangrijk. In het verleden kreeg elastine niet de aandacht die het verdient. Het gebruik van een dermaal substituut met elastine kan wondcontracturen verminderen en de elasticiteit van de huid verbeteren. Elastine-expressie is behoorlijk verminderd in littekenweefsel en nieuwe elastinevezels zijn dun, gefragmenteerd en minder gematureerd dan elastische vezels in de normale huid. Elastinevezels zullen nooit de dikte of maturatiegeraad van een gezonde huid bereiken, zelfs niet na tien jaar, wat resulteert in harde en inelastische littekens. Elastine is niet alleen functioneel maar ook ruimtelijk gedesorganiseerd in littekenweefsel. Er wordt gesuggereerd dat het gebruik van dermale substituten die zowel collageen als elastine bevatten, de aanmaak van elastine door fibroblasten kan verhogen en de verlorebn elastische vezels kan vervangen. Dit is niet het geval voor dermale substituten die geen humane elastine netwerk hebben, zoals Integra®.

Glyaderm® bevat een humaan elastine dermaal netwerk met de intacte ruimtelijke structuur van de normale menselijke huid, waardoor het mogelijke de volgende stap is op weg naar ontwikkeling van een ideale dermaal substituut.

Na de incubatietijd van zes weken wordt waterstofchloride (HCl) toegevoegd om het natriumhydroxide te neutraliseren. De van cellen ontdane huid (Glyaderm®) wordt vervolgens gespoeld in een met fosfaat gebufferde zoutoplossing, waarna het opgeslagen kan worden in 85% glycerol totdat gebruikt kan worden. Er is geen speciale opslagruimte nodig.

Studies om Glyaderm® te ontwikkelen en te valideren

De donorhuid werd gedurende verschillende tijdsperioden in NaOH geïncubeerd; 2, 4, 6 of 8 weken.

Deze dermale matrix-prototypen werden geanalyseerd met behulp van standaard histologietechnieken.

De prototypen werden vervolgens getest in een subcutaan implantaatmodel van de rat en in een varkenstransplantatiemodel; de prototypes werden geplaatst in excisiewonden van volledige dikte bedekt met autologe huidtransplantaten

Een incubatieperiode van 6 weken bleek het meest optimaal, langere perioden veroorzaakten schade aan de collageenvezels.

We zagen dat de Elastinevezels goed geconserveerd waren. Alle prototypes vertoonden intacte biocompatibiliteit in het rattenmodel door de aanwezigheid van ingroeiende bloedvaten en fibroblasten 4 weken na implantatie. Bij de prototypes die slechts 2 of 4 weken met NaOH werden behandeld, werd een ontstekingsreactie waargenomen. De prototypes behandeld met 6 of 8 weken NaOH waren in staat wondcontractie in het varkensmodel te verminderen. In de neodermis van deze wonden konden 8 weken na de operatie elastinevezels worden waargenomen die afkomstig waren van het prototype, omgeven door meer willekeurig georiënteerde collageenvezels. We zagen dat vanuit glycerol geconserveerde donorhuid een effectieve dermale matrix verkregen kon worden. Verdere klinische studies werden gepland om dit materiaal te testen op dermale substitutie in diepe (brand)wonden (**Hoofdstuk 2).**

Een varkenswondmodel werd vervolgens gebruikt om reeds bekende acellulaire dermale substituten te vergelijken met ons nieuwe prototype (Glyaderm) bereid uit met glycerol geconserveerde menselijke huid. Alle donorcellen werden verwijderd door incubatie in een oplossing van 0,06 M NaOH. De dermale substituten werden aangebracht op wonden van volledige dikte en bedekt met een STSG. Ter controle werden wonden bedekt met alleen een STSG. De reactie op wondgenezing werd gedurende 8 weken macroscopisch en op biopten geanalyseerd.

In deze tweede reeks experimenten werd Glyaderm® toegepast in een procedure in twee fasen in vergelijking met Integra®. Een week later werd de STSG op de huidvervangers geplaatst.

In de eerste serie waren de ontstekingsreactie en de instroom van myofibroblasten in Glyaderm® beperkt, wat wijst op mogelijke gunstige resultaten op de uiteindelijke resultaten van wondgenezing. De overleving van de STSG op de acellulaire dermis was lager in vergelijking met de controlewonden. Tweede serie: de opname van de STSG was hetzelfde als bij de controles, maar bovendien was de contractie van de wond verminderd. De toepassing van Glyaderm® was niet inferieur aan Integra®. Conclusie: Glyaderm® kan met succes worden gebruikt voor het verminderen van wondcontractie wanneer het wordt toegepast in een procedure in twee fasen **(Hoofdstuk 3).**

Deze "Glyaderm" DRM werd verder geëvalueerd in onze daaropvolgende studie voor bedekking van buikwanddefecten en om zo de integriteit en biocompatibiliteit ervan te testen.

Buikwandreparatie kan worden uitgevoerd met synthetische of biologische matrices. Biologische materialen kunnen het risico op infecties en fibrose verminderen. Het doel van deze studie was om twee acellulaire menselijke dermisproducten te evalueren. Een rattenmodel werd gebruikt om de twee materialen te vergelijken. Eén werd bereid met lage concentraties NaOH; het andere materiaal was SureDerm®, dat in de handel verkrijgbaar is. Defecten van volledige dikte werden in de buikwand geprepareerd en met deze matrices gesloten. Ratten werden 1 of 4 maanden na de operatie opgeofferd en het aantal verklevingen aan de ingewanden werd genoteerd. Er werden monsters genomen voor histologische analyse en om de tensiele sterkte te meten.

In beide groepen werd een goede functionele integratie van de implantaten met de buikwand waargenomen. Bij de groep met het NaOH-prototype (Glyaderm) was er geen verkleving met de darmen. In de SureDerm®-groep vertoonden 4 van de 7 ratten slechts kleine verklevingen 4 maanden na de operatie. De tensiele kracht van het genezen weefsel was 4 maanden na de operatie significant hoger in de NaOH-prototypegroep ($p < 0,0026$). De resultaten geven aan dat beide humane acellulaire dermis producten kunnen worden gebruikt in klinische studies voor het sluiten van buikwanddefecten **(Hoofdstuk 4).**

Vervolgens gingen we op zoek naar literatuur over huidvervanging bij brandwonden. Het doel van dit onderzoek was om een overzicht te geven van welke soorten huidsubstituten ontwikkeld zijn en welke vragen hieromtrent nog moeten worden beantwoord. Geen van de gecommercialiseerde producten kan momenteel claimen de optimale dermale substituut te zij vooral omdat klinisch bewijs te schaars is. Het aantal producten dat gecommercialiseerd wordt neemt niettemin gestaag toe, wat pleit voor een zeker overzicht, classificatie en duidelijke vergelijking van de beschikbare producten **(Hoofdstuk 5).**

Vanwege de relatief hoge prevalentie van ongunstige littekenvorming na brandwonden, zijn de meeste onderzoeken naar littekenbeoordeling en littekenbehandeling gericht op het brandwondenlitteken. Chirurgische en dermatologische littekens zullen gelukkig zelden resulteren in uitgebreide littekenvorming, en aangezien de impact van littekencomplicaties sterk correleert met de afmeting van het litteken (bijv. pijn, jeuk en kwetsbaarheid), is de impact van dit

soort littekens meestal beperkter, hoewel ook minder goed bestudeerd. Daarom zijn brandwondenlittekens waarschijnlijk de littekens met de grootste impact op de kwaliteit van leven. Zowel fysieke als psychische effecten die samenhangen met overmatige littekens kunnen de kwaliteit van leven belemmeren, inclusief de vaak langdurige, pijnlijke behandeling, met vaak toch een suboptimaal resultaat tot gevolg. Littekens kunnen pijn, jeuk en ongemak veroorzaken; en contracturen kunnen ook de mobiliteit beperken. De integratie van patiënten met hypertrofische littekens in een samenleving waar welzijn, individualiteit en uiterlijke verschijning steeds belangrijker zijn geworden, kan ook lastig zijn. Door veel auteurs is aangetoond dat brandwondenlittekens, vanwege hun duidelijk zichtbare en stigmatiserende uiterlijk, een grote psychologische impact kunnen hebben, vergelijkbaar met andere chronische dermatologische aandoeningen. Hoewel littekenbeoordeling essentieel lijkt, is dit nog steeds een verwaarloosd gebied en is er nog steeds geen consensus over de ideale methode voor littekenbeoordeling, ondanks de vele schalen en hulpmiddelen die de afgelopen decennia zijn ontwikkeld. Adequate beoordeling van littekens is echter belangrijk bij de klinische evaluatie en follow-up, maar het is ook essentieel om verschillende wond- of littekenbehandelingsmodaliteiten te vergelijken. Bovendien kan om medisch-juridische redenen een objectieve littekenevaluatie vereist zijn, bijvoorbeeld voor de terugbetaling van behandelingen en het bewijs van invaliditeit.

Littekenevaluatie kan worden uitgevoerd door vrij eenvoudige littekenschalen van papier en potlood die verschillende variabelen beoordelen, meestal door puur subjectieve woordbeschrijvingen (rood, verheven, enz.), maar ook door technisch geavanceerde en objectieve apparaten (littekentools) te analyseren een of meer variabelen op een meer reproduceerbare manier (spectrometrie, ultrageluid enz.). Het doel van ons onderzoek was om een analyse en een kritisch overzicht te geven van welke littekenschalen zijn ontwikkeld om de littekens te beoordelen op esthetische en fysieke aspecten van brandwondenlittekens, en wat hun rol is bij de beoordeling van brandwonden

Het gebrek aan literatuur over littekenhulpmiddelen die beschikbaar zijn voor littekenbeoordeling bracht ons ertoe om de beschikbare littekenhulpmiddelen te onderzoeken die kunnen worden gebruikt bij de beoordeling van brandwonden en littekens onderzoek **(Hoofdstuk 6 en 7)**.

Onze eerste klinische publicatie van Glyaderm® toonde gunstige langetermijnresultaten bij 55 patiënten in een procedure in twee fasen.

We zijn meer dan twee decennia geleden begonnen met het ontwikkelen van een dermaal substituut op basis van glycerol geconserveerde allotransplantaten, dat de volgende belangrijke voordelen zou hebben: humaan collageen- en elastinematrix, gemakkelijke opslag en hantering, inactivering van virussen en micro-organismen en het allerbelangrijkste: een non-profitproduct dat voor een groter aantal patiënten beschikbaar zou kunnen zijn. Als clinici in het veld was ons belangrijkste doel het ontwikkelen van een praktische en betaalbare huidvervanger voor slachtoffers van brandwonden, kanker en trauma's.

Het meest gunstige prototype Glyaderm® werd getest in dierstudies, die gunstige resultaten lieten zien in een procedure in drie fasen, allograft, Glyaderm®, autograft. Deze veelbelovende resultaten vormden de aanleiding voor de huidige pilotstudie en gerandomiseerde vergelijking.

Er zijn vele onderzoeken geweest die de voordelen van verschillende DRM's bevestigen. Voor zover wij weten, is er echter geen overtuigende gerandomiseerde studie geweest die een superieur resultaat aantoont van huidrestoratie met een dermaal substituut en huidtransplantaat boven huidrestoratie met alleen een huidtransplantaat. De meeste brandwondenexperts twijfelen niet aan de waarde van dermale substitutie bij chirurgische brandwondenzorg en de langetermijnresultaten van patiënten bevestigen de toegevoegde waarde.

Objectieve littekenbeoordeling en langere follow-up verhelderen dit voordeel, dat al klinisch duidelijk is. Onze "pilotstudie" toonde consistente, stabiele langetermijnresultaten na 6 jaar met een soepele huid na dubbellaags huidherstel met Glyaderm®. Objectieve littekenbeoordeling toonde een significant verbeterde elasticiteit van de huid bij patiënten die werden behandeld met Glyaderm® en huidtransplantaat in vergelijking met alleen huidtransplantaat ($p = 0,003$).

Glyaderm® is de eerste kosteneffectieve, niet-commerciële dermale vervanger die kan worden vergeleken met de momenteel verkrijgbare dermale equivalenten.

Een nadeel in onze eerste studies met Glyaderm® was de noodzaak van drie procedures voor volledige wondsluiting.

Directe toepassing van Glyaderm® op het wondbed zonder preparatie van een wondbed met allografts leek geen haalbare optie te zijn in zowel de dierstudies als de fase I pilotstudie. Dit werd aangetoond door onderzoek bij 3 patiënten met een volledige dikte huiddefect na het oogsten van een radiale onderarmflap. Na een-staps toepassing van Glyaderm®, verwachtten we geen problemen gezien het gezonde wondbed, maar uiteindelijk was er geen ingroei van Glyaderm®. De dierstudies hadden er ook op gewezen dat gelijktijdige toepassing van ons vroege Glyaderm®-prototype en autotransplantaat niet voldoende haalbaar was. Bij Glyaderm®-verwerking blijft een relatief dicht elastine-collageennetwerk behouden. Ontluikende capillairen moeten dit netwerk binnendringen voordat ze de bovenliggende autograft kunnen voeden. Bovendien waren de eerdere Glyaderm®-prototypes relatief te dik en hadden ze last van "batch-to-batch-inconsistenties" die inherent zijn aan variatie in selectie. Voortschrijdend onderzoek en inzicht, monitoring van selectie en ontwikkeling verbeterden dit proces van transplantaatselectie en standaardisatie. Een speciaal ontworpen "lasertool" wordt nu gebruikt om de selectie van dermis van uniforme dikte te verzekeren. De laser scant nauwkeurig de afstand tussen de optiek en de tafel en de optiek en de Glyaderm® worden vervolgens op de tafel geplaatst, waarbij het hoogteverschil gelijk is aan de dikte.

De optimale met glycerol geconserveerde dermis met een dikte van 0,2–0,4 mm wordt tegenwoordig in de EuroTissueBank geselecteerd voor verwerking tot Glyaderm®. We toonden in deze lange termijn studie aan dat Glyaderm® kan worden aangebracht met gelijktijdige huidtransplantatie na wondbedpreparatie met

allotransplantaten gedurende 5 dagen. Deze verbetering heeft een duidelijk gunstig effect op morbiditeit en kosten (**Hoofdstuk 8).**

We waren verrast om te lezen dat De Hennau et al onlangs (2021) meldden dat deze gelijktijdige transplantatie van onze vroege klinische studie de eerste was, maar blij om te zien dat onze bevindingen reproduceerbaar zijn door andere centra, wat ook onze bedoeling is. Dit centrum dat Glyaderm® sinds 2017 als DRM gebruikt, ontdekte, vergelijkbaar met onze resultaten, dat deze procedure resulteerde in een uitstekend gemiddeld opnamepercentage van 98%. In tegenstelling tot ons protocol werd de dubbellaagse huidreconstructie uitgevoerd met en zonder Negative Pressure Wound Therapy (NPWT), wat beide resulteerde in gunstige resultaten.

Samenwerking met onderzoekers van de Universiteit van Nijmegen resulteerde in visualisatie met histochemische technieken van nieuw gesynthetiseerde collageen-elastinematrix *in vitro* en *in vivo* met Glyaderm®-implantatie **(Hoofdstuk 9).**

Ten slotte voerden we "Een prospectieve, gecontroleerde, gerandomiseerde, intra-individuele vergelijkende, enkelblinde studie uit in een monocentrische setting, waarin de gelijktijdige toepassing van Glyaderm® " + autologe huidtransplantaten (STSG) werd onderzocht versus autologe huidtransplantaten (STSG) alleen in volledige dikte huiddefecten volledige en diepe brandwonden.

In dit intra-individuele onderzoek werden in totaal 66 patiënten opgenomen, wat overeenkomt met 82 wondvergelijkingen.

De gelijktijdige toepassing van Glyaderm® en autologe huid bleek niet inferieur aan het vorige protocol in termen van transplantaatopname, subjectieve littekenschubben en littekenkleur. De procedure in twee stappen bleek superieur te zijn in termen van elasticiteit. De visuele littekenevaluatie door de experts een jaar na wondsluiting was duidelijk in het voordeel van Glyaderm® bij gebruik van de tweestapsprocedure. Dit was ook het geval bij het gebruik van de gelijktijdige toepassing, maar niet zo onderscheidend als bij de procedure in twee stappen. Hoewel we geen eenduidige cijfers kunnen noemen, zijn de kosten ongetwijfeld in het voordeel van de gelijktijdige toepassing van Glyaderm® met STSG in 1 operatie.

In de handel verkrijgbare dermale substituten hebben vaak te maken met verminderde weefselvascularisatie en -integratie. Ontluikende capillairen kunnen moeilijk de DRMK penetreren wanneer ze te dicht zijn. Adequate vascularisatie vereist kostbare tijd, waardoor onmiddellijke autotransplantatie wordt voorkomen. Vaak gebruikte DRM's zoals Integra® Bilayer en Matriderm® Bilayer hebben een autotransplantatie-interval van drie weken. Dit resulteert in een verlengde ontstekingsfase, waardoor de kans op fibrose en littekenretractie toeneemt. Het autotransplantatie-interval brengt een verhoogd infectierisico met zich mee, en dit is bewezen met Integra® Bilayer. Zowel Integra® als Matriderm® hebben een enkellaags product van 1,00 mm ontwikkeld dat een procedure in één fase mogelijk maakt. Glyaderm® heeft een uniek humane collageen-elastine matrix met een dikte van 0,30 mm en is gemakkelijk te vasculariseren, waardoor onmiddellijke autotransplantatie mogelijk is . Door complicaties ging niets van de Glyaderm® of autologe huid verloren. De overleving van het huidtransplantaat was uitstekend en consistent, wat wijst op de vorming van een dermo-epidermische overgang. De biopsieën toonde adequate vascularisatie door

talrijke haarvaten. Concluderend, Glyaderm® wordt gemakkelijk en adequaat gevasculariseerd, waardoor gelijktijdige STSG autotransplantatie mogelijk is.

Veel van de biopsieën van de met Glyaderm® behandelde wondplaatsen toonden de aanwezigheid van elastische vezels aan en de meeste van deze vezels waren georganiseerd volgens een bewaard gebleven netwerkpatroon van natuurlijke vezels. Zelfs in de biopten die een jaar na wondsluiting werden genomen, konden donorelastinevezels worden gedetecteerd. Dit suggereert dat de levensduur van de elastische donorvezels langer is dan 3 maanden en waarschijnlijk zelfs langer is dan een jaar. We schatten dat het donorcollageen zou zijn verwijderd tegen de tijd dat de eerste biopsie werd verkregen. Ook al is het nog steeds een belangrijk element in het 3D-collageen-elastine-netwerk, zoals in de inleiding werd gesteld. Als de fibroblasten dit elastische netwerk als een matrix kunnen gebruiken, zou dit resulteren in een veel gunstigere oriëntatie van het littekenweefsel.

De microbiologische analyse van de wonduitstrijkjes die tijdens het onderzoek werden afgenomen, liet geen toename van de bacteriële belasting zien. Dit suggereert dat het risico op infectie niet verhoogd is. De objectieve evaluatie van de littekenkleur bij langdurige follow-up heeft aangetoond dat erytheem en pigmentatie niet vergelijkbaar zijn met die van een normale huid. De huid van de interventiegroep vertoont lichte hypopigmentatie en toegenomen erytheem. Het transepidermaal vochtverlies en de huidhydratatie van de met Glyaderm® behandelde littekens waren vergelijkbaar met die van een normale huid. Dit zijn wenselijke kenmerken van een functionele huidvervanging, waaruit geconcludeerd kan worden dat de gelijktijdige dubbellaagse reconstructie van de huid met behulp van Glyaderm® heeft geresulteerd in het herstel van de natuurlijke barrière van de huid, waardoor de patiënt wordt beschermd tegen gevaar, dwz onderkoeling, infectie en uitdroging.

Huidsubstituten zijn gebruikt bij de behandeling van verschillende medische aandoeningen. Brandwonden zijn echter een speciaal soort indicatie. Bij acute brandwonden zijn vaak grote gebieden betrokken, wat resulteert in een beperkt aanbod van levensvatbare autologe donorhuid. Bovendien wordt de situatie gecompliceerd door intense lokale en systemische ontsteking en is er slechts een klein tijdsinterval voor interventie om littekenvorming te minimaliseren. Dit was echter het resultaat van het gebruik van de tweestapsprocedure. Deze studie is de eerste grote gerandomiseerde klinische studie om de gelijktijdige dubbellaagse reconstructie van de huid met behulp van Glyaderm® te onderzoeken.

Deze studie onderzocht de toepasbaarheid van Glyaderm® bij brandwonden in een acute setting. Verschillende dermale vervangingsmiddelen, zoals Integra®, zijn met succes gebruikt bij de reconstructie van chronische brandwondencontracturen. Matriderm®, Integra® en Renoskin® kunnen worden gebruikt voor de behandeling van patiënten met blootliggend bot of pezen. In deze ernstige gevallen is het aanbrengen van STSG onvoldoende. We rapporteerden ook over het succesvolle gebruik van Glyaderm® bij een geval van brandwonden gecompliceerd door blootstelling van het scheenbeenbot na het mislukken van een vrije flapoperatie. In geselecteerde gevallen kan Glyaderm®, gecombineerd met negatieve

wonddruktherapie en huidtransplantatie, worden gebruikt als alternatief voor lapchirurgie.

De verscheidenheid aan dermale substituten is enorm en er wordt op verschillende manieren hiernaar onderzoek verricht, wat resulteert in verschillende resultaten bij elk product. Of de gelijktijdige toepassing kan worden verbeterd door gebruik te maken van dermale substitutie met Glyaderm® met een dikte van meer dan 0,30 mm, moet aandacht krijgen in toekomstig onderzoek.

Heel interessant was dat de allerbelangrijkste parameter, de algemene ervaring en gevoel van de patiënt zoals gescoord door het gebruik van de POSAS, in het voordeel was van Glyaderm® en het verschil in nam toe ten gunste van Glyaderm® groep met elk follow-up moment.

Onze onderzoeksgroep is van mening dat de littekenkwaliteit zelfs tot twee jaar na volledige wondsluiting moet worden beoordeeld. Wij zijn van mening dat het uiteindelijke resultaat in littekenkwaliteit ten minste 1,5 jaar na wondsluiting moet zijn en bij voorkeur zelfs tot 2 jaar, wanneer de weefselremodellering en onze standaardbehandelingen voor littekentherapie zijn voltooid **(Hoofdstuk 10).**

Conclusie

In de afgelopen twee decennia hebben we ons gericht op de behandeling van grote huiddefecte door Glyaderm® te gebruiken als een dermaal substituut.

Dermale substituten hebben te maken met bijzondere uitdagingen, zoals het onvermogen om adequate temperatuurbeheersing of druksensatie te bieden, verminderde vascularisatie als gevolg van langdurige overleving van de vervanger, onvoldoende immuunregulatie, mislukte integratie, hoge kosten, trage wondgenezing, infectie, pijn en onesthetische littekens. De huidige beschikbare cellulaire huidsubstituten bestaan uit slechts twee celtypes: fibroblasten en keratinocyten. Deze huidvervangingen zijn daarom niet in staat om gespecialiseerde structuren zoals klieren of haarzakjes te vormen. Er is de afgelopen jaren veel vernieuwend onderzoek gepubliceerd. Een recente studie leverde de eerste op LGR6+-stamcellen gebaseerde huidvervanger op die in staat is tot epithelisatie, haargroei en angiogenese in wondbedden. Ter illustratie van een goed voorbeeld van innovatieve ontdekking.

Een recente studie definieerde de ideale huidvervanging als volgt: "Een ideale huidvervanging zou echter een duurzame dubbellaagse reconstructie zijn die morfologisch en biochemisch vergelijkbaar is met de oorspronkelijke huid, waarbij de textuur, structuur en het vermogen om te regenereren worden nagebootst".

Op dit moment is er geen product dat aan deze hoge eisen kan voldoen. Glyaderm® bevat echter een natuurlijk dermaal netwerk dat de intacte ruimtelijke structuur van de normale menselijke huid heeft, waardoor het in theorie de ideale huidvervanger het dichtst benadert. In het eerste deel van dit proefschrift zijn de duidelijke voordelen van het gebruik van donorhuid van menselijke oorsprong genoemd. Het gebruik van van mensen afkomstige dermale vervangingsmiddelen heeft nadelen, zoals een beperkt aanbod van donorhuid, mogelijke ethische problemen, langzamere

penetratie van endotheelcellen en het ontbreken van huidaanhangsels. Er wordt al vele jaren onderzoek gedaan en er is belangrijke vooruitgang geboekt. Technologieën die ooit als "de toekomst" werden beschouwd, doen hun intrede. Electrospinning, recombinante eiwitten, engineering van kleine moleculen, autologe gekweekte huidvervangers met stamcellen en driedimensionale bioprinting zijn slechts enkele voorbeelden van de moderne benadering in de zorg voor brandwonden.

Zoals eerder vermeld, dragen brandwonden aanzienlijk bij aan de mortaliteit en morbiditeit van de bevolking wereldwijd. In 2004 waren dat bijna 11 miljoen mensen ernstig verbrand en vereisten klinisch medische behandeling. Slachtoffers van brandwonden na een trauma blijven achter met cosmetische misvormingen, verminderde functies, psychologische trauma's, problemen met dagelijkse activiteiten en sociale dysfunctie. Het doel van brandwondenzorg is, zoals altijd, niet alleen de reconstructie van het beschadigde weefsel, maar het volledige herstel van de patiënt als geheel. Slechts een van de vele mogelijke oplossingen om dit te bereiken, is het gebruik van een dermaal substituut. De resultaten van onze onderzoeken hebben ons interessante gegevens opgeleverd. We ontdekten niet alleen dat de gelijktijdige toepassing van Glyaderm® en STSG mogelijk was, maar dat de elastinevezels van de donor histologisch detecteerbaar waren, zelfs één jaar nadat de wond volledig was gesloten. De dubbellaagse reconstructie met Glyaderm® deed in veel opzichten niet onder voor zowel de gouden standaard als de tweestapsprocedure. Bovendien had het weefsel dat met Glyaderm® werd gereconstrueerd tal van kenmerken die leken op die van een gezonde menselijke huid.

Het is onze bedoeling om het toepassingsgebied van Glyaderm® als DRM voor plastisch chirurgen en brandwondenchirurgen te demonstreren.

Glyaderm® is met succes gebruikt voor andere indicaties dan diepe brandwonden en brandwondenlittekens, dwz oncologische resecties, reconstructies van donorplaatsen met vrije flap, melanocytische reuzennaevi en reconstructies van post-necrotiserende fasciitis.

We blijven ons inzetten voor ons oorspronkelijke doel en voornemen om Glyaderm® DRM beschikbaar te maken voor wijdverspreide toepassing bij brandwonden. Daartoe heeft een sterke samenwerking met plastisch chirurgen in Colombia ertoe geleid dat Glyaderm® met succes wordt geproduceerd bij de Bogota Skin Bank en in één fase en twee procedures wordt toegepast bij ernstige (aangezichts)brandwonden. Ook zorgen royalty's van boeken- en liefdadigheidsorganisaties voor financiering om Glyaderm® beschikbaar te maken voor patiënten met ernstige brandwonden en traumatische defecten over de volledige dikte.

Er moet nu en in de toekomst een scala aan onderzoek worden gedaan totdat de perfecte kant-en-klare huidvervanging en acellulaire matrix beschikbaar komen.

Glyaderm® is een praktisch toepasbare, niet-commerciële dermale regeneratiematrix en kan nu en in de nabije toekomst het kwaliteit van leven van vele slachtoffers van trauma en brandwonden helpen te verbeteren.

Hiernaast is Glyaderm® een biologische dermale matrix voor verdere celregeneratie en tissueengineering onderzoek.

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You always helped and believed in me and encouraged me to carry on with my career and life dreams. As a true friend you stood by me in the difficult years of personal relationship issues and in particular in 2010 when I almost died in an avalanche and lost my job, house and ex-wife which turned out to be a blessing in disguise. I enjoy spending time with you and your lovely wife during the conferences we attend and exchange ideas and our passions.

Dear Professor Monstrey, dear Stan,

You have been a guiding star and source of inspiration for me since I saw you at the ISBI burns meeting in 2000. “What do I have to do to be trained by you” I asked, and you told me that I had to be extremely good so there was a slim chance to make but I could always try. That is what you told me more than 20 years ago.

During my residency you constantly had to bail me out difficult political situations with your alpha male staff members who did not always appreciate my ambitions and yes, I was slow at operating. I tell my patients now that nobody will give medals for finishing 5 minutes earlier but meticulous attention to detail is the hallmark of a good plastic surgeon.

My founding year at the Queen Victoria Hospital in East Grinstead had instilled the passion for burn surgery in me and you have always pushed me to mold ideas into action and hypotheses into scientific papers.

The countless hours I have spent with you and Henk in our work and travels I will always cherish. You are a true gentleman with a passion for the finer things in life and I am privileged that I can say that you have been the pivotal factor in my career. This thesis is the culmination of the work of our research group initiated some 20 years ago.

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Dr Hoekstra, dear Hans,

The "father" of the glycerol preservation method. You always have the most elaborate ideas bordering genius. Thank you for supporting me in the early years of my career and teaching me all the experimental tips and tricks needed to test the prototypes which ultimately became Glyaderm®. You also showed me that not all those with a PhD where brilliant scientists and how to navigate the mazes of fundraising for research.

Dr Richters, dear Nelleke,

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The continuation of the Glyaderm® studies after my departure from Gent must largely be credited to your stamina.

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My buddy and paranimf Arjan,

We have been friends since 1989 and for many years share the passion for wave kitesurfing n but have been through many adventures together. I can count on you for fun and support always. You are a true friend, and I will never forget how you helped me and consoled me when I was in pieces in hospital.

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My friend Richard,

I thought I didn't need a personal trainer until I met you and we have become close friends. Thank you for taking care of me and my family.

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My colleague and friend Colin,

Thank you for your support throughout the years. I really enjoy spending time with you in the O.R., on the slopes and in the gym. You combine true grit with solid British scientific grounding and superb technical skills with a twist of dry humor. I love it.

My lovely assistants Helma, Machtelt and Marietta,

Thank you for showing up every day and putting up with me, who always changes things.

And last but not least, my family.

My dear sister Lili,

I hope you forgive me for being such a tease when we were kids. You and both your children Isabeau and Luca are very dear to my heart.

My dear father Farrokh,

The love and encouragement you have given me throughout my life has enabled me to overcome all the challenges until now.
You taught me to aim for the stars and always believe in myself and you are a most kind and gentle soul. I love you daddy.

My dear mother Susan,

We have a most special bond as being so alike and yet so different we regularly clash. You taught me discipline and to never, ever give up on my dreams. You sacrificed so much to give us a better life and education. You are strong and smart, and I am proud to be your son. Love you mummy.

My wife, my Love, Eva

Words cannot describe my love for you and our darling daughter Julie. You keep me grounded, you are my sunshine, you complete me. I love you and how you take care of our family and work as a beautiful, kind, intelligent and feisty lioness.

Curriculum Vitae

Ali Pirayesh was born in Tehran, Persia (Iran) on 22 November 1969.

His family immigrated to The Hague, Netherlands where he attended primary and secondary school (Gymnasium Haganum). He studied Medicine at Leiden University where he graduated *Cum Laude* in 1997. His fascination for plastic surgery began during an oculo-plastic research fellowship in Utah, USA.

He spent 4 years as a surgical resident in the United Kingdom where he obtained the Diploma of The Royal College of Surgeons of London in 2000. His fascination with burn care began whilst working at the Queen Victoria Hospital and Burn Unit in East Grinstead.

This hospital was commissioned by Queen Victoria to treat the horrible burn wounds of the RAF pilots during the Battle of Britain at the second world war.

He started his plastic surgery residency at the Gent University Hospital in 2001. During his first year he started experimental animal research in wound healing of burns in Amsterdam.

The idea to develop an artificial dermis from donor skin was based of the daily challenges he saw in surgical burn reconstruction in the Gent Burns Unit.

The support of his mentor Professor Stan Monstrey, Henk Hoeksema and research co-workers Nelleke Richters and Hans Hoekstra made it possible to obtain various grants to develop the first non-commercial dermal substitute Glyaderm®.

He completed his residency in plastic surgery and gained further experience in (Aesthetic) plastic surgery working in hospitals and private clinics in Brazil, United Kingdom and The Netherlands.

He began to work as an independent Plastic Surgeon within his own private clinic Amsterdam Plastic Surgery in 2008.

Ali Pirayesh is an internationally renowned plastic surgeon and a sought-after global instructor and lecturer in aesthetic plastic surgery and regenerative medicine.

His specialist interest includes burn & tissue engineering research and regenerative medicine, aesthetic surgery of the face, breast and body contouring and non-surgical (facial) rejuvenation.

He is the President of the Netherlands Society of Aesthetic Plastic Surgery (NVEPC) and past Executive Board member of the Netherlands Society of Plastic Surgery (NVPC).

He is the Scientific Director of Aesthetic Multispecialty Society.

Ali is married to Eva, and they live with their darling daughter Julie in Amsterdam.