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Transcriptional Analysis of Normal and Pathological Scars

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Annika Sophie Walter

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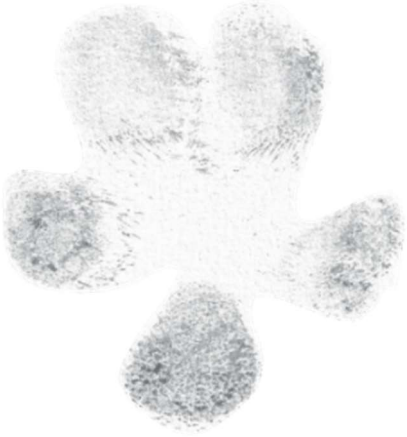
Do what you feel in your heart to be right- for you'll be criticized anyway.

You'll be damned if you do, and damned if you don't.

(Eleanor Roosevelt)



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Content

List of publications

Papers as basis of the dissertation:

Article I:

Walter A.S., Stocks M., Akova-Ölken E., Gauglitz G., Hartmann D., Aszodi A., Böcker W., Saller M.M., Volkmer E.; Keloids are transcriptionally distinct from normal and hypertrophic scars. *European Journal of Dermatology*. 2023.

Article II:

Stocks M., Walter A.S., Akova-Ölken E., Gauglitz G., Aszodi A., Böcker W., Saller M.M., Volkmer E.; RNA-seq unravels distinct expression profiles of Keloids and Dupuytren's disease. *Heliyon*. 2024.

Further publications during the time as doctorate candidate:

Article III: Attachment A:

Walter A.S., Volkmer E., Gauglitz G., Böcker W., Saller M.M.; Systematic Review of Molecular Pathways in Burn Wound Healing. *Burns*. 2023.

Individual scientific contributions

For my thesis I engaged in interdisciplinary research about two similar fibrotic diseases in the human body in the field of hand and plastic surgery: I. wound healing and skin scar formation and II. genetic determined fibrotic diseases such as Dupuytren`s disease.

I. Contribution to Article I: Keloids are transcriptionally distinct from normal and hypertrophic scars

I chose to research how skin wound healing and pathological wound healing differs genetically, with comparison of normal scars, hypertrophic scars and keloid scars. Over a period of 8 months, I specifically collected and prepared skin scar samples from hand and plastic surgeries. I collected 43 normal and pathological skin scar samples after hand and plastic surgery in cooperation with PD Dr. med. Elias Volkmer, Prof. Dr. med. Georg Gauglitz (Studienzentrum Prof. Dr. med. Gauglitz) and Prof. Dr. med. Daniela Hartmann. I was supported by PD Dr. rer. nat. Attila Aszódi as head of the musculoskeletal research center (MUM) LMU, and Dr. rer. nat. Maximilian Saller as former leader of the research group “molecular interactions of neural and musculoskeletal systems” (MUM, LMU). They helped with the elaboration of the final study design and introduced me to clinical experts and other research groups, such as the Gene Center Munich LMU which supported the gene sequencing of the tissue samples used in my research.

I began my research by experimenting with five different protocols for RNA-Isolation of fibrotic tissue. From this, I identified and detected for my research the protocol with the highest amount and best quality of RNA for further Bulk-RNA Sequencing

This step of my research was supported by Marcus Stocks who assisted me with literature search and technical implementation support. Martina Burggraf (MUM, LMU) supported this research by further RNA-processing and library preparation. Next-generation sequencing of 12 normal scar samples, 3 hypertrophic scar samples and 3 keloid scar samples was carried out with support of the Gene Center Munich LMU. Dr. rer. nat. Maximilian Saller and Elif Akova-Ölken gave support for further complex bioinformatic analysis.

I wrote Article I independently with revisions by co-authors.[1] Marcus Stocks shares first authorship in this article thanks to his active support in identifiying the research protocol about gene sequencing of fibrotic tissue and by writing the manuscript section “materials and methods”.[1]

As part of the research project, I created a questionnaire for the affected patients and secured their consent, which includes the anonymous use of photos (Figure 4) and the use of skin tissue. I used the programs Microsoft Excel® and R® for analysis and graphic visualization, for the statistical evaluation of the patients´ questionnaire. I wrote and graphically

designed this cumulative thesis, using Biorender® with literature search to create scientific illustrations (Figure 1-3).

II. Contribution to Article II: RNA-seq unravels distinct expression profiles of keloids and Dupuytren's disease

This second research project explores similarities and differences in gene expression in keloid skin scars and Dupuytren´s disease tissue.[2] I collected fresh tissue samples after surgical removal with support of PD Dr. med. Elias Volkmer, Prof. Dr. med. Hartmann and Marcus Stocks. Finally, I collected 3 keloid skin scar samples and Marcus Stocks collected 3 samples of Dupuytren´s disease tissue. Based on our experiences with different experimental protocols for RNA isolation and next-generation sequencing of scar tissue (Article I), I prepared the tissue samples of keloid scars and Dupuytren´s disease similar to our first project.[1] Martina Burggraf (MUM, LMU) supported this research by further RNA-processing and library preparation. My bioinformatic analysis was supplemented by additional data about Dupyutren´s disease tissue from Marcus Stocks. Dr. rer. nat. Maximilian Saller and Elif Akova-Ölken supported us with evaluation and visualization of our bioinformatic results. To get more information about keloid skin scars with their clinical peculiarities and specialties in treatment, I exchanged professional information with Prof. Dr. med. Georg Gauglitz, a specialist in dermatology and dermatological research (Studienzentrum Prof. Dr. med. Gauglitz), as well as PD Dr. Elias Volkmer, a hand surgeon. I wrote most of Article II with support from Marcus Stocks for the materials and methods as well as the discussion part.[2] Marcus Stocks developed the idea of expanding our first research project with collecting Dupuytren´s disease tissue after hand surgery. Because of Marcus Stocks' technical and content support, the first authorship is shared.[2]

As part of the research project, I created a questionnaire for the affected patients and secured their consent, to include the anonymous use of photos (Figure 4) and skin tissue. I used the programs Microsoft Excel® and R® for analysis and graphic visualization for the statistical evaluation of the patients´ questionnaire. I wrote and graphically designed this cumulative thesis, using Biorender® with literature search to create scientific illustrations (Figure 1-3).

1 Introduction

1.1 Human skin

1.1.1 Structure of human skin

The average middle-aged male with a body weight of 70kg contains on average 30kg (43%)[3] of muscle mass, 7kg (10%)[3] of skeletal mass (excluding bone marrow) and 6.1kg (8,7%)[3] to 10.5kg (15%)[4] of skin with subcutaneous tissue. The skin is the third heaviest organ and the organ with the largest surface area in the human body about 1.73 m^2.[5] Depending on the proportion of subcutaneous fat and muscles, the skin can assume larger dimensions and adapt to conditions by stretching.

Microscopically, the skin can be differentiated between cutis, which can be subdivided into epidermis and dermis, and subcutaneous tissue (Figure 1A). [4,6,7]

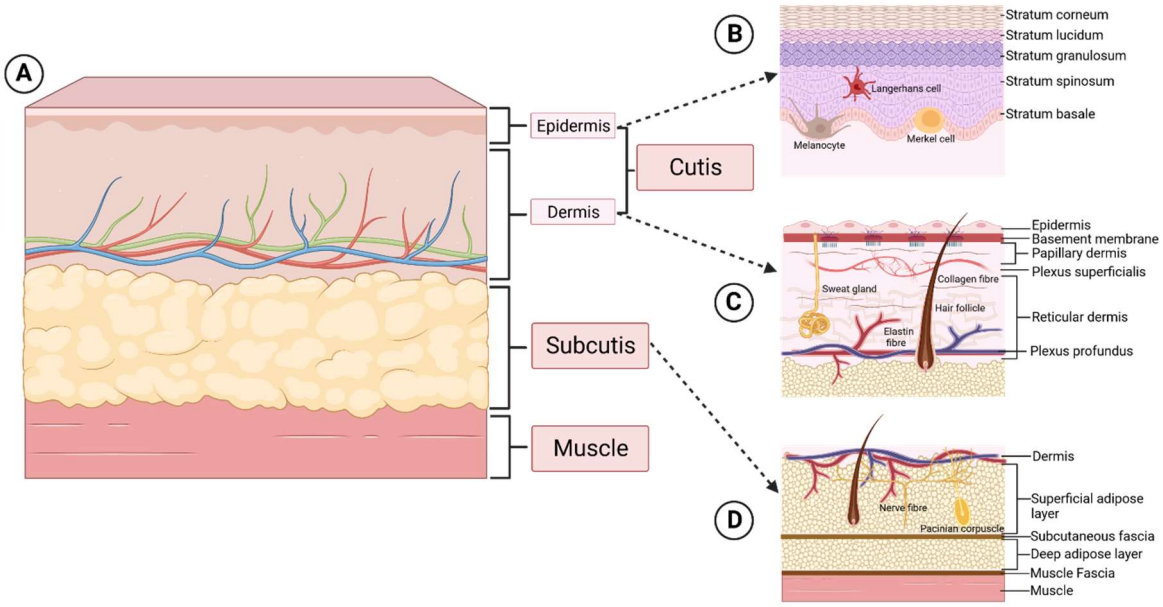

Figure 1: Anatomy of human skin
A illustrates the anatomical subdivision of human skin with cutis and subcutis and the underlaying muscle tissue. **B**: sublayers of the epidermis. **C**: anatomical regions of the dermis. **D**: layers of the subcutis. Created with Biorender®.[4,6,7]

The epidermis (Figure 1B) is the thinnest layer of the skin. It is a vascular free, outward protective skin layer that consists mainly of keratinocytes in various stages of differentiation. Depending on the differentiation process of these keratinocytes, five sublayers can be distinguished.[7–9] The epidermis also contains melanocytes, Merkel cells and Langerhans

cells.[8] The most abundant protein in the epidermis is keratin, a structural protein for cytoskeleton in epithelial cells, hair, nails and callused skin.[10]

The dermis (Figure 1C) is the middle layer of skin. It is thicker than the epidermis and has vasculature and connective tissue.[9] Epidermis and dermis are tightly connected through dermal-epidermal junctions, which form the basement membrane zone of the skin. This zone is characterized by a specific molecular composition and protein interactions.[10,11] Two sublayers of the dermis can be distinguished: papillary dermis and the deeper reticular dermis. The papillary dermis is a thin, collagen and elastin fiber-rich layer of loose connective tissue containing fibroblasts, adipocytes, immune cells and nerve endings.[4,9] A capillary mesh (Plexus superficialis) with small blood vessels, appears to be the transition between papillary and reticular dermis.[9] The reticular dermis is a dense connective tissue with thick collagen fibrils and the surrounding elastic fibers. Due to its firm fibrous structure, the reticular dermis forms the outline of the skin. The reticular dermis anchors skin appendages such as hair follicles, sebaceous and sweat glands, and contains lymphatic cells, adipocytes and nerves. Bordering the subcutaneous connective tissue, a complex capillary system (Plexus profundus) can be found.[6,9,12]

The lowest layer of the skin is the subcutaneous connective tissue (also named as subcutis or hypodermis), (Figure 1D).[7,9,13] In this layer of skin, the loose connective tissue, which is made up from collagen fibrils and elastin, is intersected by adipose tissue, epifascial veins and nerves.[14] For perceiving vibrations, mechanoreceptors called Vater-Pacini corpuscle or Pacinian corpuscle are embedded.[10]

1.1.2 Function of human skin

The skin protects the body from environmental influences in several ways. It protects against UV radiation through synthesis of melanin, it regulates body temperature through transpiration, like sweating, and protects against allergenic agents, chemicals and mechanical irritation. Additionally, the skin performs metabolic functions during vitamin D synthesis and has multiple sense organs such as sense of touch and of temperature, as well as of nociception.[9,14] Due to the skin's contact with potential pathogens, the skin has several immunological functions such as a physical barrier with antimicrobial peptides and lipids, which defend the skin while destroying bacterial membranes.[15] The normal pH of human skin is 5.4-5.9, in order to avoid bacterial infections.[16] Several immune cells like Langerhans cells, dendritic cells and macrophages reside permanently in the skin.[15]

1.2 Wound healing phases and scar formation

Due to its permanent contact with potential damaging environmental influences, the skin has developed multiple regenerative functions. Injuries that destroy skin cells and the integrity of the skin coat trigger the wound healing process. Wound healing is a complex biological process designed to stop bleeding, reduce the risk of infections and restore the best possible functional condition.[17–20] After bleeding and hemostasis, there are three wound healing phases (Figure 2): inflammation phase, proliferation phase and remodelling phase.[18–22]

Wound healing is regulated by multiple factors such as proteins, cytokines, hormones, oxygen levels, bacterial infections and the interactions of different cell types.[18,21,23] During wound healing, multiple biomolecular factors are differently expressed, either for only one wound healing phase or over multiple wound healing phases.[19,22] Because there is a smooth transition between the wound healing phases, it is difficult to make a strict assignment of these molecular regulators to a particular phase.[19,20,23,24] (Figure 2). Figure 2 shows the expression of selected cell types and proteins within the wound healing phases. These regulation processes depend on the type of injury, depth and expansion of injury, cell hypoxia, dehydration, chronic wound disorders and other patient individual factors, that present with pre-existing illness (for example Diabetes mellitus).[19,23,25]

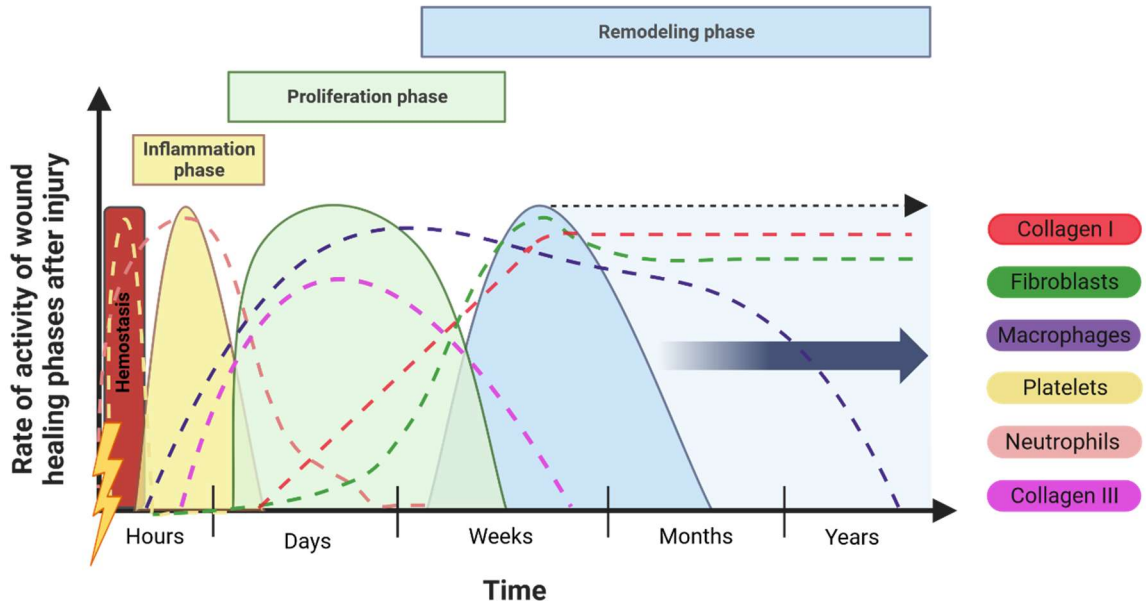

Figure 2: Wound healing phases
commencing with injury and hemostasis followed by inflammation phase, proliferation phase and remodelling phase. Overlapping expression of cell types and biomolecules. Created with Biorender®.[19,20,23,24]

The duration of each wound healing phase in humans is difficult to define as patients demonstrate individual temporal variations.

The inflammation wound healing phase (day 1- 6)[26] (Figure 3.1) starts directly after bleeding stops, with hemostasis, vasoconstriction and building a thrombocyte aggregation plot with fibrin.[18,21] Neutrophile granulocytes and lymphocytes immigrate to break down bacteria and dead cells.[15,18] Monocytes start differentiation to phagocytic macrophages.[25] Wound secretion from blood and lymph fluid moisturizes and rinses the wound.

During the proliferative wound healing phase, macrophages, fibroblasts and keratinocytes fill in the wound with granulation tissue (Figure 3.2).[21,23,27] Literature documents the duration of the proliferation phase as between 4-14[26,28] days, expanding up to 4-21 days[29]. In the proliferation phase the re-epithelization and collagen synthesis with ECM remodelling starts. To create a new vascular network angiogenesis, with high rates of blood vessels compared to uninjured skin, start to ensure the supply of oxygen and nutrients and removal of waste products.[18,30] The further the healing process of the wound progresses, the more the individual wound healing phases overlap, with the aim to close the wound edges and to construct a tissue that comes functionally close to the original uninjured skin.[27,29]

While the proliferation phase is active with increased re-epithelization and ECM organization, the remodelling phase (Figure 3.3) starts with connective tissue re-organization and increased tissue contraction with myofibroblasts.[29] The remodelling phase overlaps with the proliferative phase and typically starts 14 days after injury.[28] Scar formation and tissue remodelling can take several months to years.[21,28,29]

In uninjured skin, the amount of Collagen-III fibres is greater than the amount of Collagen-I fibres, which develop from Collagen-III fibres.[31–33] Importantly, the remodelling phase of wound healing of injured skin, the rate of changes of collagen fibres from Collagen-III (increased expression during proliferation phase) to Collagen-I, is highly increased in pathological fibrotic processes such as scar formation (Figure 2).[31–33] After deep injuries with destruction of multiple cell layers, the newly formed collagen fibres are not able to form cross-links between themselves, which causes reduced elasticity of the newly formed scar tissue.[26,31,34] Due to increasing fluid loss, the wound dries out and the tissue begins to contract. Additionally, melanocytes are not able to regenerate, and this is followed by hypopigmentation of the new scar. Histopathologically, scars show a loss of their skin appendages, like hair follicles and glandes.[24] This remodelling process can take multiple weeks to months, and in the case of severe injuries, the tissue can still be restructured years later.[18,20,31,34,35] Ideally, this results in a normal scar (Figure 3.3, Figure 4a) that is white, thin, unobtrusive and not painful, without limitations in movement. With the conclusion of re-epithelialization

and formation of the scar tissue, the initial wound healing process is officially finished. Scar formation is a complex process and still ongoing.

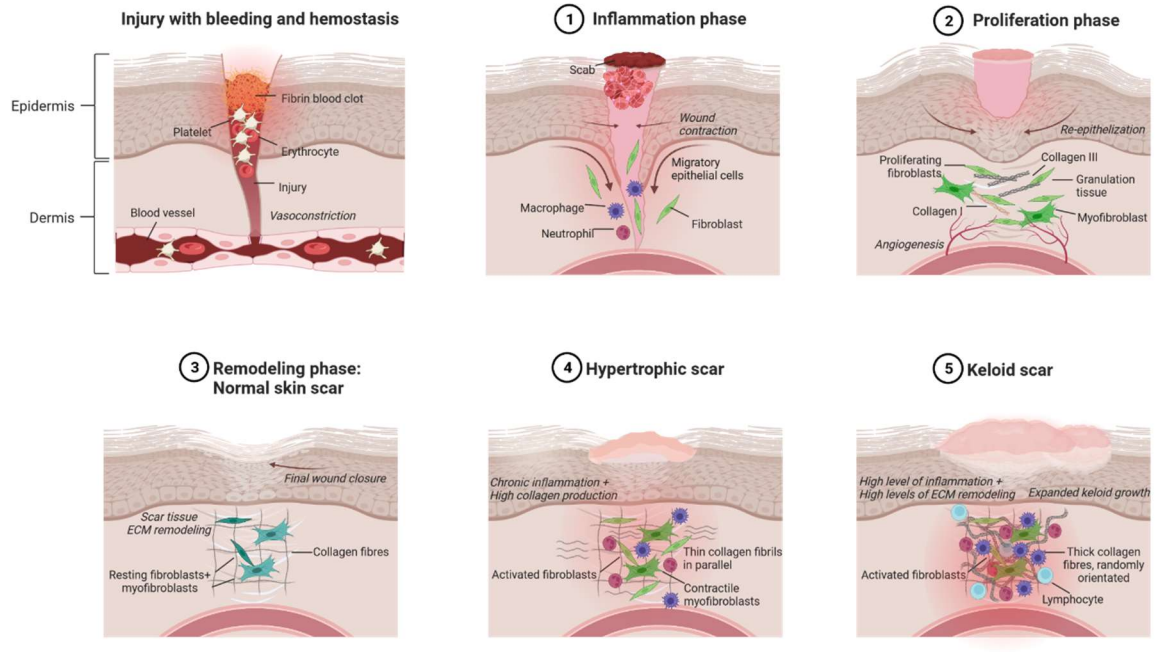

Figure 3: Demonstration of physiological and pathological skin wound healing bleeding and hemostasis. **3.1**: inflammation phase. **3.2**: proliferation phase. **3.3**: remodelling phase with normal skin scar formation. **3.4**: hypertrophic scar formation. **3.5**: keloid scar formation. Created with Biorender®.[18,21,23,27,29]

1.3 Wound healing disorders and pathological scar formation

Complex biological processes can be easily thrown out of balance in each wound healing phase. Pathological wound healing with formation of wound healing disorders is an increasingly common problem in healthcare.[35,36] Delayed wound healing, especially disturbances during the inflammation or proliferative phases, can develop into a chronic wound healing disorder which can cause subsequent medical problems.[20,26] Genetic conditions and disturbances during the proliferative or remodelling phases with increased expression of inflammatory modulators can lead to pathological scar formation. Pathological scars such as hypertrophic scars or keloid scars show a disturbed structure of connective tissue (Figure 3.4, 3.5).[20,26,37,38] Wound healing disorders and pathological skin scars have far-reaching physical and psychological limitations for patients in daily life.[36] Chronic wound disorders with non-healing skin wounds are estimated to cost more than $3 billion per year in the health care system of the United States.[20,39,40]

1.3.1 Causes and risk factors for pathological wound healing

Wound healing and scar formation is influenced by multiple individual factors. Most significant is the type of injury (cut, burn, contused laceration) and patient genetic variations.[35,36] Other important factors are mechanical tension, infections, early effective medical care, patient individual genetic factors and pre-illness conditions.[26,34,35,40] Circulatory disorders like peripheral artery disease and chronic venous insufficiency, diabetes mellitus, polyneuropathy, malnutrition, immunosuppression, smoking and age can cause delayed and pathological wound healing. These conditions also create a high risk for developing chronic ulcerations and pathological wound disorders, for example diabetic foot syndrome and pathological scar formation.[17,20,26,40] Pathological skin scars differ from normal scars by changes in color, thickness, sublimation and spreading beyond the edges of the wound, which may cause aesthetic dissatisfaction and limitation of movement.[35,36] Hypertrophic scars and keloid scars show signs of chronic inflammation, excessive angiogenesis and changes in collagen remodelling in the reticular dermis.[41] In the context of this multifactorial influenced biological process, wound healing is prone to interfere with subsequent pathological final results. The presence of comorbidities, genetic variations, the emotional impact and social stigma of these scars and limited efficacity of long-acting treatment with disruption of daily activities, can make the Treatment of pathological scars complex and challenging.[26,35,36]

1.3.2 Included types of pathological skin scars

Skin scars can be categorized based on their haptics, pigmentation and colouring, spreading and localisation, affect the formation of the scar essentially.[35,36] In addition to normal and inconspicuous scars, a distinction is made between hypertrophic scars, keloid scars and atrophic scars. My research focuses on hypertrophic scars and keloid scars, two fibroproliferative disorders,[41] and compares them to normal skin scars and fibrotic tissue of M. Dupuytren (Figure 4A).

Hypertrophic scars (Figure 4B) often occur after burning, accidents, traumatic surgeries or other skin diseases (acne, folliculitis). They are challenged by high tensile and pressure loads on the tissue.[38,42] These scars often occur in direct temporal relation with wound healing in the first six months after injury with gradual regression over a period of a few years.[35,43] They show a thick, padded and erythematosus, partially hyperpigmented, scar formation. Hypertrophic scars are limited in their expansion to the original wound borders.[35,43,44] In addition to the aesthetic abnormalities, the patients report pain, dysesthesia and movement limitations, followed by an increase of morbidity.[44,45] Hypertrophic scars can spontaneously regress over time.[35,43,44]

Keloid scars (Figure 4C) mostly have a familiar genetic condition and are more common in families with darker skin tone or Asian or African origin.[46] For the most part keloid scars develop after small traumata, partially superficial skin wounds, insect bites and cut wounds including surgical wounds.[35,42] The sternum, head, neck area and earlobes are predisposed to develop keloid scars.[46,43] Usually the differentiation to keloid scars starts several weeks to months after finishing the primary wound healing process.[44,47] Clinically, keloid scars present with over proliferating thick, nodular connective tissue. They often show changes in colouring and growth beyond the initial wound borders with partially new splotches of keloid tissue in the surrounding area of the initial injury.[35,43,44,46] Due to their characteristics spreading, keloid scars are often categorized as benign tumours.[47] A lot of patients report touch sensitivity and itching.[45,46] In contrast to hypertrophic scars, keloid scars show no potential for spontaneous regression.[35,44]

There are a variety of different treatment options for hypertrophic scars and keloid scars which must be discussed with the affected patients.[35,41,43]

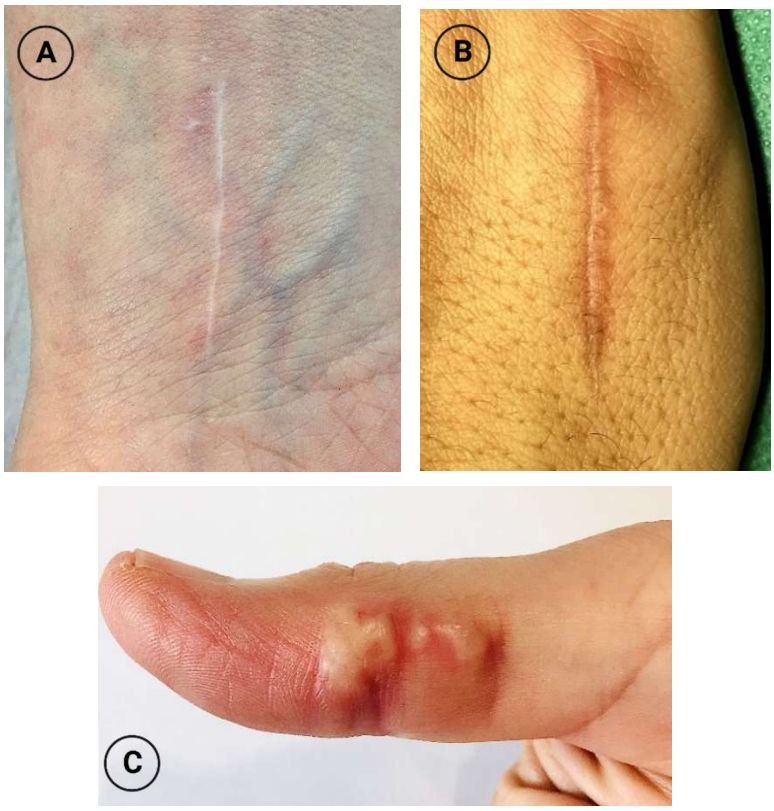

Figure 4: Normal and pathological skin scars

A: Normal skin scar after hand surgery, **B**: Hypertrophic skin scar, **C**: Keloid skin scar.

1.3.3 Other fibrotic disorders in human body on the example of Dupuytren´s disease

Regeneration of destroyed tissue is a complex biological processes. The human body works to repair damaged tissue with the best results in functionality and in the shortest time. Fibrotic processes are part of the normal biological wound healing and scar formation.[48,51] Fibrotic processes are defined by accumulation of extracellular matrix, in particular fibrous connective tissue using collagen fibres and glycosaminoglycan.[48,49] In and around fibrotic tissue, there are high levels of inflammation and different expressed cell types like macrophages, fibroblasts, contractile myofibroblasts. Lymphocytes and mast cells can be proven.[49] During the different wound healing phases and fibrosis development, different molecular factors such as cytokines, growth factors and angiogenic factors for example TGFB1, PDGF, VEGF, CTGF and IL-13 will be poured out.[49,50] In case of normal skin scarring, fibrotic remodelling is a controlled part of wound healing and ends with completion of the remodelling phase. Fibrotic processes can also be found in every part of the human body, especially after infections in and around the organs (for example lung fibrosis, cardiac fibrosis or liver fibrosis) or in fibroproliferative disorders such as Dupuytren´s disease.[48,49,52] Compared to fibrotic normal scarring after skin wound healing, which is limited in expansion, fibrotic organ scarring, pathological skin scarring or scarring due to genetic conditions can expand. Fibrotic remodelling processes can occur in organs and cause a permanent scarring, organ malfunction and can be life-limiting.[48,49]

Dupuytren´s disease is a genetic condition with formation of nodular, fibrotic hardening of connective tissue strands mainly concerning the palmar aponeurosis of the hand.[52] It affects around 8% of the general Western population.[52] Untreated patients show an individual chronic progression of contraction in bending the fingers. In particular, the palmar side of the fingers IV and V are concerned, often in both hands.[53] Symptoms include movement limitations, pain, and irritations of nerves and vessels. Dupuytren´s disease shows a prevalence of 8% worldwide with increased occurrence in Africa (17%) and Asia (15%) compared to Europe (10%) and the United States (2%).[54] Men are affected 3-4 times more often than women, with increased occurrence over the age of 50.[54] There are ectopic manifestations of this disease known as Morbus Ledderhose and Induratio penis plastica.[55,56] Fibrotic diseases are influenced by multiple individual genetic factors, age and environmental risk factors.[53] Conservative as well as operative treatments, may be available depending on severity of symptoms and progression of the disease.

2 Research project: Transcriptional Analysis of Normal and Pathological Scars

In 2018, I began to think about research topics that involve interdisciplinary molecular research about a central medical problem widespread in the population. With the support of PD Dr.rer.nat. Attila Aszodi, Dr.rer.nat. Maxmilian Saller and PD Dr.med. Elias Volkmer, I decided to focus on the broad field of wound healing disorders with focus on scar formation and fibrotic processes in the field of hand and plastic surgery.

2.1 Construction and main goals of the research project

To gain basic theoretical content, I engaged in an immense literature search about molecular pathways in burn wound healing and did so in cooperation with Dr. rer. nat. Maximilian Saller.[38] Burn wound healing often results in hypertrophic scar formation with longtime physical and psychological restrictions for patients concerned.[57] This research led to our creation of the first interactive map for visualization of molecular interactions during burn wound healing with assignment to wound healing phases. This is available on Wikipathways, with worldwide open-free access. We also created interactive illustrations about molecular players and their known interactions during burn wound healing in mammals, separated for four species: Homo sapiens, Mus musculus, Rattus norvegicus and Sus scofa (Attachment A). We merged our results and visualized them via Cytoscape. This interactive map shows the current state of known molecular interactions during burn wound healing with assignment to wound healing phases and gives new starting points for further research (Attachment A). Additionally, we identified important gene hallmarks during burn wound healing. Our final results are published as a systematic review (Attachment A).[38]

Next, for the experimental part of this research project, I collected normal and pathological skin scar samples during hand surgeries. Scar tissue belongs to fibrotic tissue in the human body. We decided to expand our research project and started a cooperation with Marcus Stocks to include Dupuytren´s disease tissue as another type of fibrotic tissue in the field of hand surgery. The next step was the establishment of an experimental protocol for RNA isolation of scar tissue. For this purpose, I conducted a series of five different experimental protocols to process fresh fibrotic tissue. For better results, I started the preparation of tissue directly on site in the operating room. The protocols varied with the size of tissue samples, storage liquids and ambient temperature. In the laboratory, the RNA isolation was carried out with the support of Martina Burggraf and was checked for quality using the Bioanalyzer at the Gene Centre Munich LMU. The fibrotic tissue samples with the highest RNA-Integrity number (RIN) were included for library preparation and next-generation sequencing. The data was then bioinformatically evaluated and proceeded graphically to present gene

expression differences between the included different fibrotic tissues.[1,2] This step was supported by Elif Akova-Ölken. As part of the doctoral project, I fulfilled all the mandatory requirements of the Munich Medical Research School.

2.2 Research questions, hypothesis and aims of the studies

Fibrotic tissue is characterized by chronic inflammation processes and changes in histological composition of connective tissue with, among other things, changes in types of collagen fibres, numbers of fibroblasts and contractile myofibroblasts, without the occurrence of hair appendages such as hair follicles and glades.[48,58] During wound healing and scar formation, the type of produced collagen changes.[59] In uninjured skin, the amount of Collagen-III fibres is higher than Collagen-I fibres.[31] In the proliferation phase, the synthesis of Collagen-III is a major extracellular matrix component in different organs, particularly in skin.[59,60] During the remodelling phase of wound healing, the amount of Collagen-III decreases and the synthesis of Collagen-I is greatly increases, especially in pathological fibrotic processes (Figure 2).[31,59] Collagen-I occurs, for example, in bones, fibrocartilage and tight connective tissue such as fascia.[61,62] The ratio of Collagen-I to Collagen-III in normal scars is 6:1, while keloid scars show a ratio of 17:1.[44,47] These histological findings can be reconciled with the clinical characteristics of keloids. The collagen synthesis in keloids is three times higher than in hypertrophic scars.[43] To get additional transcriptional data next to known histological knowledge about fibrotic tissue, we performed next-generation sequencing with subsequent bioinformatic analysis. With the help of bioinformatic data sets and freely available DESeq1data sets, we were able to uncover potential gene expression differences between normal, hypertrophic and keloid skin scars as well as between keloid scars and Dupuytren´s tissue.

2.2.1 Article I: Keloids are transcriptionally distinct from normal and hypertrophic scars

Based on our first systematic review about molecular interactions during burn wound healing with subsequent hypertrophic scarring[38], we designed an experimental set-up to uncover gene expression similarities and differences. This included visualization of potential clustering between normal scar samples, hypertrophic scar samples and keloid scar samples of human skin.[1]

We hypothesized that normal scars, hypertrophic scars and keloid scars show different gene expression profiles in Bulk-RNA sequencing.

As part of the project, I collected 37 normal skin scar samples, 3 keloid skin scar samples and 3 hypertrophic skin scar samples during hand surgeries. After implementing the

experimental protocol for RNA-Isolation of fibrotic scar tissue and performing the next-generation sequencing, a bioinformatical analysis was performed.

To identify and visualize the gene expression differences with potential clustering between different skin scars, an LDA-Analysis and Venn diagrams were created. Additionally, the top ten differentially expressed genes and MA plots of different scar types in comparison were designed. Finally, we created a heatmap of the top 50 expressed genes sorted by scar type, to visualize potential common clustering. To figure out the connections between gene expression and regulation of biological pathways, a Gene-Ontology (GO) pathway analysis for keloid scars was performed. This gained molecular genetic knowledge can be used for verification of clinical classification of these pathological scar types.

2.2.2 Article II: RNA-seq unravels distinct expression profiles of keloids and Dupuytren's disease

In our second study, we compared gene expression profiles of keloid skin scar samples to Dupuytren´s tissue after hand surgery.[2] Both fibrotic diseases show clinical similarities such as tough, hard and inelastic scar tissue which can restrict movement and use of the hand.[43,52,54] Both fibrotic conditions show a potential chronic progression with accompanied sensory disturbances and increased aesthetic dissatisfactions.

Due to their clinical and microscopic similarities, we hypothesize that keloid scars and Dupuytren´s disease show similar gene expression profiles in Bulk-RNA sequencing. During hand surgeries, we collected 3 keloid skin scar samples and 10 Dupuytren´s disease tissue samples in collaboration with Marcus Stocks. We sought to provide a bioinformatical analysis of gene expression differences between the two types of tissue and create different figures for visualization of potential molecular connections. We first performed a principal component analysis using regularized DESeq2 data, Venn diagram and Ma plots. The top 100 differentially expressed genes in Keloid scar tissue and Dupuytren´s disease tissue were displayed in a heatmap. We also identified the top ten most important genes per disease which appear to be of central importance in disease development. This may provide useful starting points for the development of new therapeutic agents. For visualization and networking of protein-protein-interactions (PPI), a PPI network analysis of DEGs was carried out. Our goal was to reveal some important protein interactions in the pathogenesis of Dupuytren's disease and keloid formation. To better understand the regulation of biological processes during fibrotic tissue development, we used bioinformatical data to construct a GO pathway analysis as well as a KEGG pathway analysis of upregulated and downregulated biological processes.

3 Article I: Keloids are transcriptionally distinct from normal and hypertrophic scars

Investigative report

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Keloids are transcriptionally distinct from normal and hypertrophic scars

Background: Wound healing and skin regeneration after injury are complex biological processes, and deep injuries with a high degree of tissue destruction may result in severe scar formation. Clinically, scars can be classified into normal, hypertrophic and keloid scars. However, the molecular signature of each scar type is currently not known. *Objectives:* The aim of this study was to reveal the transcriptional landscape of normal, hypertrophic and keloid skin scars following hand and plastic surgery based on total RNA sequencing. *Materials & Methods:* Eighteen skin scar samples from hand and plastic surgeries of human donors were minced directly after removal and stored in TRIzol (Thermo Fisher, USA). Samples were then subjected to RNA isolation, cDNA library preparation, bulk RNA sequencing and bioinformatics analysis. *Results:* We show that keloid scars transcriptionally differed from normal and hypertrophic scars. Normal and hypertrophic scars presented overlapping clustering, and eight genes were shown to be commonly expressed between hypertrophic and normal scars. No genes were specifically expressed at a higher level in keloid and normal scars. Based on gene ontology pathway analysis, genes with a higher level of expression in keloid scars lead to increased (extra-) cellular matrix proliferation and cell interaction. Moreover, tumour-like genes were more highly expressed in keloid scars, supporting the clinical impression of strong and diffuse growth. *Conclusion:* This study furthers our understanding of the classification of differential scar types based on molecular signature, which may shed light on new diagnostic and therapeutic strategies for keloid scars in the future.

Key words: hand surgery, hypertrophic scar, keloids, RNA sequencing, skin scar, wound healing

The human body has the ability to adapt and regenerate after exceptional situations and physical damage [1]. The first damaged organ is almost always the skin [1], which is the largest organ of the human body covering an area of 1.5-2.0 m² for a 170-cm tall individual [2]. Because the skin is the body's barrier to the environment, it is often exposed to potentially harmful stimuli. Blunt and sharp objects, as well as thermal injury leads to damage of the skin and surrounding soft tissue, activating the physiological wound healing process [3, 4]. The body starts a fulminant regeneration program in order to close the wound as fast as possible and restore functionality [5, 6].

The skin can be categorized by its main layers: the epidermis, dermis and subcutaneous fat (hypodermis) lying beneath. There are four stages of wound healing after trauma or injury: the haemostasis phase, inflammation phase, proliferation phase and regeneration phase with scar formation [6-8]. Exterior injury can be extensive and deep, damaging many cells and layers of the skin. In this case, proliferation of secondary wound tissue is activated in order to fill in the cavity of the lesion [6, 7]. Different cell types, such as keratinocytes and fibroblasts, are essential for subsequent scar formation [6]. By forming fibrotic tissue, the body attempts to fix the wound, reduce the risk of infections and restore functionality [9, 10]. Hence, scar formation is the final step of wound healing and can take a long time, in some cases months [8]. Ideally, the skin forms a white-coloured, thin scar without functional impairment [11]. However, sometimes the wound healing process is disturbed and distinct pathological scars may form [12].

Such pathological skin scars can be classified into hypertrophic scars, keloids and atrophic scars, depending on multiple, partly still unknown, genetic risk factors [12, 13]. Chronic wounds, darker skin colour, localisation, medical treatments, and age are known to increase the risk of pathological scar formation [14]. Until now, diagnostic differentiation between hypertrophic scars and keloids has been based on clinical aspects [14]. A hypertrophic scar is a raised scar between the boundaries of the wound [15]. In contrast, keloids are raised scars that

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extend behind the original wound boundaries, invade originally healthy tissue, and regrow after excision surgery [13, 16, 17]. Profibrotic genetic changes are discussed as aetiology [18]. The interpretation of scars is case-dependent, resulting in different diagnoses between different examiners and, consequently, different therapeutic treatments [19, 20]. There is a lack of unambiguous genetic and biochemical markers, which could help to identify the correct scar type and the optimal therapeutic strategy. Hypertrophic scars can be treated with injections of glucocorticoids, cryotherapy, pressure, laser treatments and surgical removal [19, 21, 22]. In contrast, operative removal of keloids can increase the risk of regrowth immensely [13]. Therefore, the correct diagnosis of the scar type is necessary in order to choose the appropriate therapeutic strategy [16]. Biochemical factors and transcriptional markers could be used to aid diagnosis, and next-generation RNA sequencing of normal scars, hypertrophic scars and keloid material, as investigated here, may help in reducing errors in diagnosis and improve patient outcome.

Materials and methods

Ethics statement, sample acquisition and processing

This study was approved by the ethics commission of the Ludwig-Maximilians-University Munich (Project number 19-177). All donors were at least 18 years old and signed informed consent prior to enrolment in the study. Furthermore, all donors were of Caucasian origin and had not undergone any medical treatment, such as chemotherapy, intralesional steroid injection or radiotherapy, prior to scar removal surgery. Human scar tissues were obtained from 18 patients during implant removal or for aesthetic reasons, and were visually classified into the three main scar types independently by the surgeon and the study nurse. All scars were mature, and all aspects of the scars were used including specimens from the centre and the outer zone. Detailed patient and sample information are provided in *supplementary table 1*.

All samples were processed immediately after dissection from the patient in the operation room. The samples were minced with a scalpel in a drop of ice cold, sterile 0.9% sodium chloride solution in a Petri dish. Afterwards, tissue pieces were placed into 5-ml TRIzol solution (Thermo Fisher, USA) in a 15-ml Falcon tube and transferred to the laboratory for further processing. Scar tissues were homogenized three times at 5,000 rpm for 20 seconds using a high-throughput tissue homogenizer (Precellys 24, Bertin Technologies, France), and the homogenates were centrifuged at 12,000 rpm for 10 minutes at 4°C to remove tissue debris. Finally, the supernatants were stored at -80° C until RNA isolation.

RNA isolation, library preparation, sequencing and bioinformatic analysis

Total RNA was isolated from the TRIzol homogenates using the Direct-zol™ RNA MiniPrep Kit (Zymo Research, USA) with 96% ethanol and TRIzol column, and RNA integrity was validated using a BioAnalyzer (Agilent, USA). RNA integrity numbers (RIN) were comparable and ≥1.9 cDNA sequencing libraries were generated with the SENSE mRNA-Seq Library Prep Kit V2 (Lexogen, Austria) according to the manufacturer's protocol. All libraries were sequenced on the same run using a HiSeq1500 device (Illumina, USA) with a read length of 50 bp and a sequencing depth of approximately 20 million reads per sample. After demultiplexing the samples, reads were aligned against the human reference genome (release GRCh38.101) using STAR (version 2.7.2b) [23], and those below 10 reads per gene were filtered out. Gene expression was normalized with variance stabilizing transformation (vst), and dimensionality was reduced with linear discrimination analysis (LDA) (MASS Version. 7.3-54). DESeq2 (version 1.28.1) [24] was used for differential gene expression analysis using shrinkage estimation for dispersions and fold changes to improve stability and interpretability of estimates with a *p*-adjusted cutoff value of ≤ 0.05 and log2 fold change cutoff of ± 2. The MA plots were visualized using the ggplot2 R package. Venn diagram was created using significantly different expressed genes with a *p*-adjusted value of <0.05. Gene set enrichment analysis (GSEA) was conducted for each scar type to identify significant gene ontology (GO) biological pathways using the R package (clusterProfiler, version 3.14) [25]. The top 50 differentially expressed genes (*supplementary figure 1*) derived from DESeq2 were shown and clustered using the hierarchical average linkage clustering and Euclidean distances in the R package for Non-negative Matrix Factorization.

Results

Keloid scars have a distinct transcriptional landscape compared to normal and hypertrophic scars

Clinically, different scar types are classified according to their visual appearance and potential for continuous growth. However, there is currently no known molecular marker that separates normal, hypertrophic and keloid scars. Thus, total RNA sequencing of different scar types was performed, and expression differences were determined by DESeq2 analysis.

MA plots (*figure 1A-C*) were used to visualize gene expression data sets between two groups. Log2 fold change versus Log2 mean expression between two scar types was displayed as a scatter plot, and significantly different expression levels were identified (coloured dots). We found that genes encoding extracellular matrix proteins, such as *COL10A1* (collagen type X alpha 1 chain), *COL11A1* (collagen type XI alpha 1 chain) and *KRT6C* (keratin 6C), were significantly overexpressed in keloid scars compared to normal scars (*figure 1A*). *COL10A1* is a hypertrophic chondrocyte marker involved in endochondral ossification during skeletal development and is usually expressed at the late phase of chondrogenesis in adult mesenchymal stem cells *in vitro* [26, 27]. Mutations in *COL11A1,* an another gene

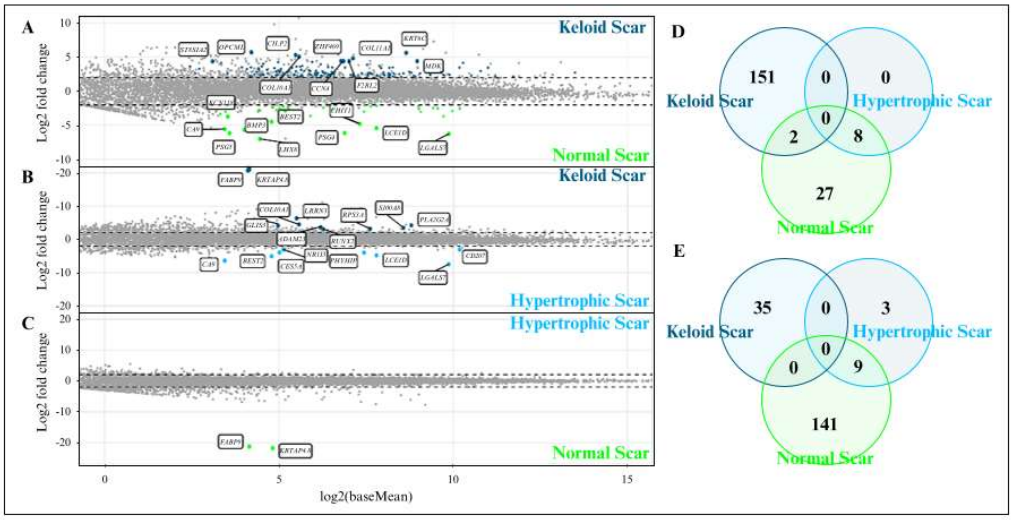

Figure 1. Gene expression in keloid scars, hypertrophic scars and normal scars. A-C) MA plots of differentially expressed genes between different scar types; colours represent significantly high gene expression in the respective scar type, and non-significant genes are marked in grey. D, E) Venn diagrams depicting genes with significantly high (D) and low (E) gene expression, which is unique or overlaps between normal, hypertrophic and keloid scars; there is no overlap of significantly regulated genes between keloid scars and hypertrophic scars.

highly expressed in cartilage, were found in human cancers including cutaneous squamous cell carcinomas (cSCC), which can increase neoplastic invasion in skin tissue [28]. Keratins, such as KRT6C, are intermediate filament proteins found in the cytoplasm of epithelial cells, in hair and nails. Keratin filaments provide the structural stability of skin cells. Mutations in *KRT6C* cause focal palmoplantar keratodermas, a characteristic feature of pachyonychia congenita [29, 30]. Down-regulated genes in keloids, compared to normal scars, included: *BMP3* (bone morphogenetic protein 3), *CA9* (carbonic anhydrase 9), *LGALS7* (galectin 7), and *LHX8* (LIM homeobox 8). LGALS7 influences cell-cell and cell-matrix interactions and is expressed in keratinocytes, which is of particular interest regarding the properties of keloid tissue [31, 32]. *Supplementary figure 2* shows different volcano plots of the different scar tissue comparisons. *Figure 1B* shows gene expression in keloid scars compared to hypertrophic scars. *LGALS7* was poorly expressed in keloid scars compared to hypertrophic scars. Interestingly, the expression level of 14 genes was significantly higher, and the expression level of nine genes was significantly lower in keloid scars compared to hypertrophic scars. The number of differentially expressed genes was higher in the keloid scar group vs. normal scar group, when compared to the two other pathological scar types. In addition, there were no genes more highly expressed in hypertrophic scars relative to normal scars. Only two genes, *FABP9* (fatty acid binding protein 9) and *KRTAP4.8* (keratin associated protein 4-8), demonstrated significantly lower expression in hypertrophic scars compared to normal scars (*figure 1C*). These results reinforce the delineation of keloid scars from other scar types at the transcriptomic level. However, normal and hypertrophic scars were not as clearly distinguishable based on transcriptional differences. Our results suggest that keloid scars have a distinct transcriptional landscape, when compared to normal or hypertrophic scars.

In *figure 1D and E*, the number of differentially expressed genes based on DeSeq2 analysis is presented in Venn diagrams. Among the total 16,216 expressed and filtered genes, 151 were more highly expressed in keloid scars in comparison to 27 genes which were more highly expressed in normal scars. This high number of up-regulated genes in keloid scars, compared to normal scars, may reflect their pathogenicity and increased gene expression rate. No genes were more highly expressed in hypertrophic scars compared to normal scars. Eight genes demonstrated significantly higher expression in normal and hypertrophic scars. These genes were: *BEST2* (bestrophin 2), *CA9* (carbonic anhydrase 9), *CD207* (CD207 molecule), *NR1I3* (nuclear receptor subfamily 1 group I member 3), *CES5A* (carboxylesterase 5A), *PHYHIP* (phytanoyl-CoA 2-hydroxylase interacting protein), *LCE1D* (late cornified envelope 1D) and *LGALS7*. Compared to keloid scars, there was more pronounced overlap in gene expression between normal and hypertrophic scars. In particular, there was no overlap between genes with significantly high or low expression levels in pathological (hypertrophic and keloid) scars.

Figure 1E shows the genes with a significantly lower expression level in the three scar types based on DeSeq2 analysis. In keloid scars, 35 genes showed a significantly lower level of expression, compared to only three genes

in hypertrophic scars. These genes were: *IGFBP3* (insulin-like growth factor binding protein 3), *KRTAP4.8* and *FABP9*. The group with the largest number of genes with a significantly low level of expression was normal scars with 141 poorly expressed genes. This result is consistent with the clinical impression of decreased deposition of extracellular matrix during normal scar formation and increased matrix deposition and remodeling in pathological scars. In addition, nine genes were significantly poorly expressed in both hypertrophic and normal scars. There was no overlap between down-regulated genes in keloid scars and normal scars. An overview of all differentially expressed genes for each comparision is provided in *supplementary table 2A-C*.

Identification of distinct classes of scar groups based on linear discriminant analysis (LDA) of gene expression

In order to classify the gene expression data between the scar groups, LDA of the top 100 genes derived from DESeq2 (*figure 2*) was performed. LDA revealed grouping of donors according to the corresponding scar type and showed clear separation between normal and keloid scars without any overlap. On the other hand, the classes of hypertrophic and normal scars partially overlapped, suggesting that these scars have a more similar gene expression pattern compared to keloid scars. A heatmap of the top 50 differentially expressed genes is presented in *supplementary figure 1*.

GO biological pathway analysis indicates activation of pathways associated with cellular and extracellular matrix organization

In order to better understand the functional aspects of differentially expressed genes between keloids and normal scars, we performed GO biological pathway analysis (*figure 3*). Hypertrophic scars and normal scars were genetically close to each other and had a lower number of differentially expressed genes. Therefore, no significant GO pathway analysis was possible. However, our data show that genes that are more highly expressed in keloid scars activate pathways related to cellular organization and extracellular matrix organization, consistent with the clinical features of keloids with increased deposition of extracellular matrix components and altered wound healing.

Discussion

Keloids and hypertrophic scars are clinically distinct pathological scars caused by cutaneous injury or irritation. The aetiology of keloidal and hypertrophic scarring is characterized by inflammation and excessive extracellular matrix deposition, but lacks reliable biomarkers for molecular stratification. Our study compares the transcriptional landscape of three scar types by RNA sequencing. We demonstrate that on the transcriptional level, the two pathological scar types, hypertrophic and keloid scars, differ moderately; keloid scars are markedly different from normal scars, and hypertrophic scars show a high degree of similarity to normal scars.

Keloid scars have a distinct transcriptomic signature compared to hypertrophic and normal scars

LDA demonstrated that keloids are clearly distinct from normal and hypertrophic scars at the transcriptional level (*figure 2*). Keloid scars are characterized by a high number of highly expressed unique genes and show no or minimal overlap of differentially expressed genes with the two other scar types (*figure 1*). The genes upregulated in keloid scars, in comparison to normal scars, include *COL10A1*, *KRT6C*, *CCN4* (cellular communication network factor 4), *CILP2* (cartilage intermediate layer protein 2) and *MDK* (midkine), suggesting activation of pathways involved in extracellular matrix proliferation and cell communication. These findings on the transcriptional level are supported by GO and GSEA shown in *figure 3* and are in line with the clinical impression of keloid scars. Midkine is a heparin-binding growth factor modulating angiogenic and proliferative cell activities, particularly during tumourigenesis [33-35]. Keloid scars excessively grow beyond the wound edges by invading adjacent tissues, thus they share characteristics with cancerous growth [36]. The increased growth of keloids, a clinically defining feature, can thus be explained by upregulation of tumour-stimulating genes such as *MDK*. The appearance of keloid tissue is harder, rougher and less elastic than hypertrophic and normal scars [36]. By upregulating *COL10A1* and *CILP2,* two chondrogenic marker genes, keloid scar tissue can be stabilized, acquire a firmer appearance and matrix stiffness can be enhanced [37, 38].

The clinical finding that keloids erupt from wound edges, while hypertrophic scars remain confined within wound boundaries has led to the assumption that keloid scars are a type of aggravated hypertrophic scar [39, 40]. Importantly, our RNA sequencing results clearly oppose the hypothesis that keloids develop from hypertrophic scars. The gene expression pattern of keloid scars differs from that of hypertrophic scars (*figure 1, 2*), suggesting that keloid scars are not merely exacerbated hypertrophic scars. *GLIS3* (GLIS family zinc finger 3), *RUNX2* (RUNX family transcription factor 2) and *TNC* (tenascin C), among others, are upregulated in keloids in comparison to hypertrophic scars (*figure 1A*). GLIS3 is a member of the GLI-similar zinc finger protein family [41, 42] and plays an important role during embryonic development and osteoblast differentiation via induction of FGF18 [41, 43, 44]. Mutations in GLIS3 are associated with neonatal diabetes and congenital hypothyroidism as well as renal cystic dysplasia, progressive liver fibrosis and osteopenia [41, 43]. RUNX2, an osteoblast-specific transcription factor, plays a role in chondrocyte maturation and osteoblast differentiation with proliferation of osteoprogenitors [45-47]. RUNX2 is known to play a role in human cancer, including bone cancers and osseous metastasis, in particular, breast cancer [48, 49]. A recent single-cell RNA-sequencing study, comparing keloids and normal scar tissue, also showed enrichment of tissue factor *RUNX2* in mesenchymal fibroblasts, suggesting an important role in fibroblast differentiation. Enrichment analysis of these mesenchymal fibroblasts revealed upregulation of genes related to osteogenesis and chondrogenesis, such as *COL11A1* and *COMP*, which is in line with our results [50].

Figure 2. Linear discriminant analysis (LDA) of gene expression classified by scar type. Coloured ellipses indicate the association of each sample with its clinical scar type based on gene sequencing. Normal scars are coloured in green, hypertrophic scars in light blue and keloid scars in dark blue. Each numbered rectangle represents a donor.

Tenascin C is an extracellular matrix protein which is highly expressed in various tissues during development, usually downregulated in adult tissues but upregulated in pathological conditions such as wound healing, neuronal degeneration, inflammation and cancer [51, 52]. Pathological scar formation is characterized by excessive fibroblast proliferation in the whole body [53-57]. TNC is highly expressed in fibroblasts and regulates fibroblast migration, thus it might be responsible for the formation of the dense fibrotic tissue in keloid scars [58, 59]. Besides its profibrotic properties, TNC acts as an endogenous activator of Toll-like receptor (TLR) 4, which is associated with inflammatory processes such as arthritic joint diseases, *e.g.* rheumatoid arthritis [60]. In arthritic joint diseases, TNC and the TLR4 it activates take a leading role in the synthesis of proinflammatory cytokines with destructive synovial inflammatory reactions [60].
GO biological pathway analysis shows that stem celllike activation with epithelial to mesenchymal transition, bone remodelling, and ossification play a role which might explain the increased, uninhibited and abnormal matrix proliferation and changes in consistency in keloids. Keloids show structural similarities to hardened cartilage tissue, and less structural similarities to healthy skin tissue. Clinically, this might explain the low response to some therapeutic strategies for keloids which are normally used in normal scar therapy.

Hypertrophic scars are similar to normal scars

Hypertrophic scars are thick, raised scars between the original wound boundary with decreased elasticity, consistency and aesthetic appearance. In contrast to keloid scars, hypertrophic scars show minimal differences in gene expression, when compared to normal scars (*figure 1C*) and display no uniquely upregulated genes (*figure 1D*). Only two genes, *FABP9* and *KRTAP4.8*, were expressed at a lower level in hypertrophic scars and high level in normal scars. FABP9 belongs to the group of fatty acid-binding proteins involved in cytosolic lipid metabolism and lipid transport, and also plays a role in metabolic and inflammatory pathways, especially in cardiovascular diseases [61, 62]. KRTAP (keratin associated protein 4.8) is part of the KAP (keratin associated protein) family [63]. It can be found in hair follicles and during nervous system development and keratinization [64]. Mutations in KRTAP4.8 were detected in trochlear nerve disease and inflammations of the vulva [64].

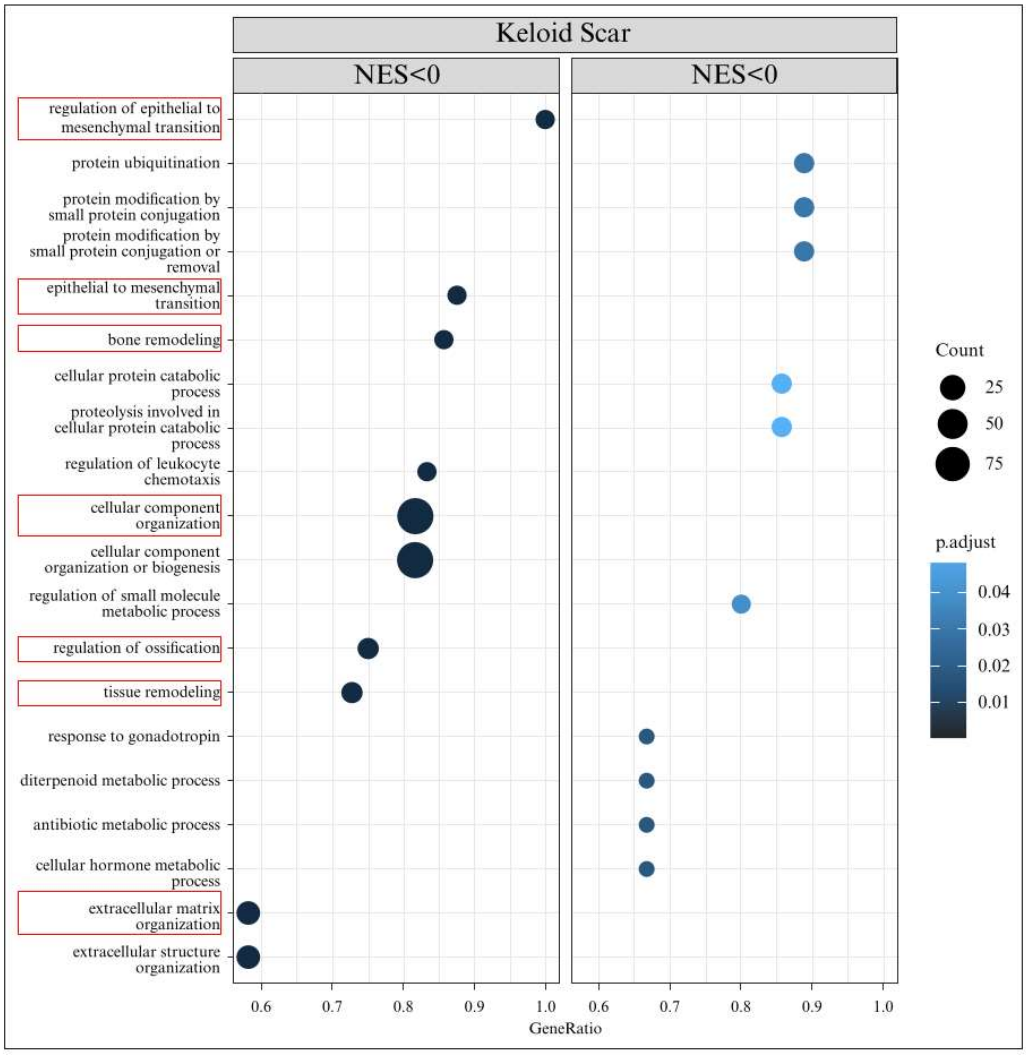

Figure 3. GO biological pathway analysis and GSEA of differentially expressed genes for keloid and normal scars. Dot size indicates the number of genes that show significantly higher or lower expression, and dot colour represents the level of significance based on adjusted p values.

Eight genes were found to be significantly upregulated in both normal and hypertrophic scars. *LGALS7* encoding galectin-7, an unglycosylated lectin binding to β-galactoside, in keratinocytes regulates cell-cell and cell-matrix interactions, apoptosis, cytokine signalling and lipid metabolism [65, 66]. *CD207* is found in skin cells, especially Langerhans cells in the epidermis and mucosa [67, 68]. *BEST2* and *CA9* are genes that encode for transmembrane proteins in different organs [69-71]. These results indicate that, at the transcriptomic level, normal and hypertrophic scars are more similar to each other than to keloid scars.

Conclusion

Keloid scars significantly differ from hypertrophic and normal scars at the transcriptional level, which supports the clinical presentation of the main types of skin scars. Hypertrophic scars transcriptionally resemble normal scars with increased extracellular matrix production and interaction. Keloids are characterised by upregulation of genes related to tumour-like and chondrocyte differentiation, features that are not observed in either hypertrophic or normal scars. Our study suggests, based on

transcriptional evidence, that keloid scars are fundamentally different from hypertrophic scars, and that hypertrophic scars are derived from normal scars. These observations have implications for therapeutic strategies and clinical management of pathological scars. Keloids that recur at a high rate after surgery may require different therapies, and molecular aspects may be potential targets for research. Further bioinformatical analysis could be improved by testing selected cohorts of similar patients selected via analysis of age, gender and time between injury and scar extraction as well as stricter anatomical restrictions on the localizations of wounds. Due to the low number of pathological scars in this study, the range of these parameters was broad. Further studies using single-cell sequencing and personalised approaches, with attention to factors causing variation, should pave the way towards novel solutions for the therapy of keloid scars. ■

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Data availability: *gene expression data and transcripts per million reads (TPMs) for all analysed samples are provided in supplementary tables 3 and 4, respectively.*

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4 Article II: RNA-seq unravels distinct expression profiles of keloids and Dupuytren's disease

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RNA-seq unravels distinct expression profiles of keloids and Dupuytren's disease

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A B S T R A C T

Keloid scars and Dupuytren's disease are two common, chronic, and incurable fibroproliferative disorders that, among other shared clinical features, may induce joint contractures. We employed bulk RNA sequencing to discern potential shared gene expression patterns and underlying pathological pathways between these two conditions. Our aim was to uncover potential molecular targets that could pave the way for novel therapeutic strategies. Differentially expressed genes (DEGs) were functionally annotated using Gene Ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with the Database for Annotation, Visualization, and Integrated Discovery (DAVID). The protein-protein-interaction (PPI) networks were constructed by using the Search Tool for the Retrieval of Interacting Genes (STRING) and Cytoscape. The Molecular Complex Detection (MCODE) plugin was used for downstream analysis of the PPI networks. A total of 1922 DEGs were identified within Dupuytren's and keloid samples, yet no overlapping gene expression profiles were detected. Significantly enriched GO terms were related to skin development and tendon formation in keloid scars and Dupuytren's disease, respectively. The PPI network analysis revealed 10 genes and the module analysis provided six protein networks, which might play an integral part in disease development. These genes, including CDH1, ERBB2, CASP3 and RPS27A, may serve as new targets for future research to develop biomarkers and/or therapeutic agents.

1. Introduction

Keloids are abnormal proliferations of scar tissue forming at the site of cutaneous injury. They may be defined as benign fibrous skin tumors with an uncontrolled cell proliferation beyond the borders of the original wound [1,2]. Dupuytren's disease is a fibroproliferative disorder of the hands associated with an uncontrolled fibroblast growth and extracellular matrix deposition [3]. Both conditions are incurable, they both tend to chronically progress and have a certain genetic predisposition [4,5]. They both share a higher prevalence in specific ethnic groups and occur in areas of high mechanical stress [2,3]. Both diseases may induce contractures. Often times, surgery is necessary to improve function of the affected limb despite not being a definite cure due to a high rate of recurrence. Fibrotic Dupuytren's nodules originate within the affected hand's diseased aponeurosis, a robust network of fibrous tissue that interconnects the skin with the underlying structures of the hand, including bones and tendon sheaths. Typically devoid of pain, these nodules tend to align along the longitudinal axes, progressively forming strands that lead to finger contractures. This process ultimately culminates in significant constraints on hand function and a marked diminishment of the patient's quality of life [5]. Both conditions share similarities on a microscopic level, as well. For one, keloids and Dupuytren's disease show an increase of fibroblasts

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with an excessive extracellular matrix deposition, particularly collagen type I and III [5,6]. It is known that molecular changes in contractile myofibroblasts and regulation of matrix proliferation play a pivotal role in the evolution of both conditions, yet the cause of the diseases is not fully understood [3,4]. New therapeutic strategies, such as pharmacological injections, radiotherapy and ultrasound were tested, but their outcomes are unsatisfactory and inferior to surgical removal of the fibrotic tissue [2,7]. Based on these pathophysiological similarities, we hypothesized that common molecular characteristics may exist between Dupuytren's disease and keloid scars. In an attempt to detect similarities on a molecular level and to identify potential molecular targets for a non-surgical, potentially definitive therapy, in this study we applied high throughput next generation RNA-sequencing to three tissue samples of either condition.

2. Materials and methods

2.1. Ethics statement, sample acquisition and processing

This study was approved by the ethical commission of the Ludwig-Maximilians-University Munich (Project number 19–177). All six donors were at least 18 years old and signed informed consent prior to enrolment in the study. Human keloid scar tissue was obtained from three patients during either implant removal or surgical scar correction for aesthetical reasons. Keloids were classified as such by the surgeon and the study nurse independently. Three samples of Dupuytren's tissue were obtained during surgical correction of finger contractures. In Supplementary Table S1 detailed patient and sample information is listed. To ensure an appropriate RNA quality of the samples, all samples were processed immediately after dissection from the patient inside the operating room. The dissected samples were minced in a drop of ice cold and sterile 0.9 % sodium chloride solution in a Petri dish with a scalpel. Afterwards, tissue pieces were transferred to 5 ml TRIzol (Thermo Fisher, USA) in a 15 ml falcon tube and transferred to the laboratory for further processing. To loosen the cell structures, the tissue pieces were homogenized three times at 5000 rpm for 20 s using a high throughput tissue homogenizer (Precellys 24, Bertin Technologies, France). Afterwards, the homogenate was centrifuged at 12,000 g for 10 min at 4 °C to remove leftover extracellular matrix pieces. Finally, the supernatant was stored in a −80 °C freezer until sequencing library preparation.

2.2. RNA library preparation, sequencing and Bioinformatic analysis

For the total RNA isolation 5 ml TRIzol-tissue-homogenates (Directzol™) and the RNA MiniPrep Kit (Zymo Research, USA) with 96 % ethanol were used according to the manufacturer's protocol. Subsequently, the RNA integrity was validated with a BioAnalyzer (Agilent, USA). Following the instructions provided by the manufacturer, RNA-sequencing libraries were generated with the SENSE mRNA-Seq Library Prep Kit V2 (Lexogen, Austria). All libraries were sequenced on the same run with a HiSeq1500 device (Illumina, USA) at a read length of 50 bp and a sequencing depth of approximately 20 million reads per sample. The next step was to demultiplex the samples with the corresponding Illumina sequencing primers. After demultiplexing, reads were aligned against the human reference genome (release GRCh38.101) using STAR (version 2.7.2b) to establish read per gene counts for 60672 genes. Before processing the differentially expressed genes, the genes that were lower than 10 reads in the total of the whole samples were filtered out by the rowSum method [8] and the analysis was performed with the remaining 22914 genes. The gene expression was normalized using the variance stabilizing transformation (vst) method and the dimensionality was reduced with a principal component analysis (PCA) [9–11]. For differential gene expression analysis, the DESeq2 package (version 1.28.1) was used with a predefined p-adjusted value cut off of $\leq$0.05 and a Log2FoldChange cut off of $\pm$2 [12]. To generate the MA plots, we employed ggpubr [13]. The Venn diagram was constructed based on genes displaying significant differential expression, characterized by a p-adjusted value $<$ 0.05. To identify significant Gene Ontology (GO) Biological Pathways, we conducted a Gene Set Enrichment Analysis (GSEA) for each condition. This was accomplished using the R package clusterProfiler (version 3.14) [14].

2.3. Functional enrichment analysis of DEGs

The list of significant DEGs was used to perform The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using the Database for Annotation Visualization and Integrated Discovery (DAVID) online tool [15,16]. Cut-off values were a p-value $<$0.05 and a gene count per annotated pathway $\geq$2.

2.4. Protein-protein-interaction (PPI) network and module analysis

The PPI network was constructed by using the Search Tool for the Retrieval of Interacting Genes (STRING) database based on all significantly up- or downregulated DEGs. A preset combined protein interaction score of $>$0.4 was selected on the STRING database as a cut-off value to construct the network. Cytoscape was used to visualize the network [17]. The biological significance of the modules was assessed using the Molecular Complex Detection (MCODE) plugin. The modules were ranked by their degree and selected with an MCODE score $>$5 and a number of nodes $>$6 [18]. A KEGG pathway enrichment analysis was conducted for the top 5 modules of the PPI network.

3. Results

3.1. Identification of DEGs and principal component analysis: keloids and Dupuytren's disease display entirely distinct expression profiles

We extracted high quality RNA from all tissue samples and identified a total of 1922 differentially expressed genes, which were statistically significant (padj. <0.05). Out of these 1922 genes, 1401 genes have been upregulated within the keloid scar samples whereas 521 genes have been upregulated within the Dupuytren's disease samples. A full list of all significantly DEGs can be found in the supplementary tables (Table S2). The principal component analysis (Fig. 1A) revealed a tight clustering among the Dupuytren samples, while greater variability was observed in the keloid group. Interestingly, despite clear clinical and microscopic similarities, we found absolutely no overlap between the two diseases. They displayed entirely distinct expression profiles. Fig. 1B depicts the Top 100 differentially expressed genes between both entities. Most of the genes belong to the family encoding for skin related processes, e.g., FLG2, which is essential for normal cell-cell adhesion and proper cornification [19]. IVL encodes the expression of the eponymous protein and contributes to the formation and protection of corneocytes [20]. In addition, it has a crucial role in normal skin, aberrant expression patterns of IVL have been found in keloid scar tissues and have been associated with increased epidermal thickness [21].

The Venn diagram in Fig. 2A shows that 1401 genes were significantly upregulated in keloid tissue and 521 genes were significantly upregulated in the Dupuytren's disease group. The diagram further shows that there was no overlap in significantly expressed genes. In total there were 13571 expressed genes. The MA plot (Fig. 2B) displays significantly and non-significantly expressed genes. In tissue samples of Dupuytren's disease several genes were significantly upregulated in comparison to keloid samples, e.g., HCN1 (Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 1; FC = 8.5, padj. < 0.05). Similarly, TREM1 (Triggering Receptor Expressed on Myeloid Cells 1; FC = 8.4, padj. < 0.05) and MYO3A (Myosin IIIA) transcripts (FC = 8.4, padj. < 0.05) were significantly upregulated. As expected, several transcripts of proteins relevant to skin related processes were significantly upregulated in keloid tissue as opposed to the Dupuytren's disease samples, namely, FLG2 (Filaggrin 2) (FC = 12.7, padj. < 0.05) and LCE2B (Late Cornified Envelope 2B) (FC = 11.4, padj. < 0.05).

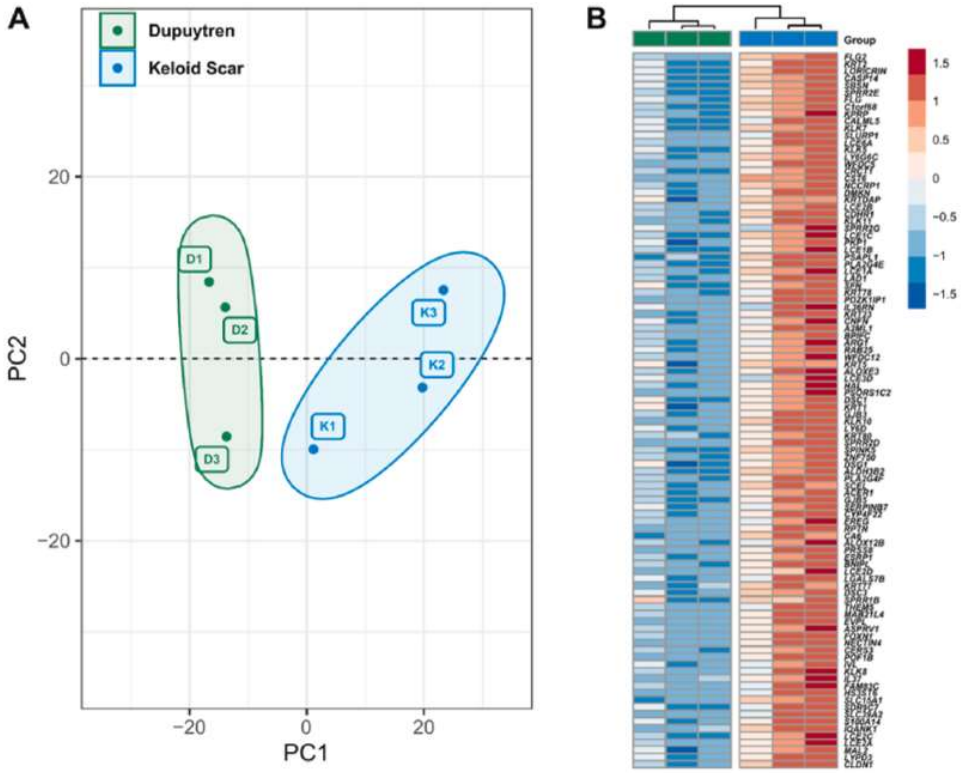

Fig. 1. A) A principal component analysis was performed on the regularized log transformed count data using DESeq2. Green dots represent the Dupuytren samples and blue dots the keloid samples. Despite clinical and microscopical similarities, there is absolutely no overlap between the two conditions. **B)** This figure represents a heatmap of differentially expressed genes between the keloid and Dupuytren samples. The Top 100 genes displayed in the map were clustered using the hierarchical average linkage clustering and Euclidean distances in the R package for Nonnegative Matrix Factorization.

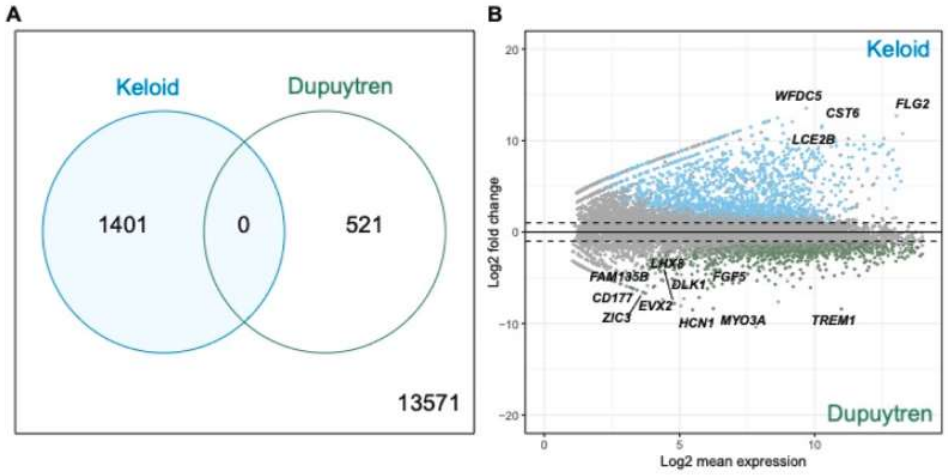

Fig. 2. A) The Venn-Diagram displays all differentially expressed genes and shows no overlap between Dupuytren's disease and keloid scar tissue samples. In total 13571 genes were expressed. **B)** The MA plot illustrates the upregulated genes of keloid scar and Dupuytren's disease samples. The data points above the x-axis depict upregulated genes in keloid scars, whereas data points below the x-axis show upregulated genes in Dupuytren tissue samples. Blue dots indicate significantly upregulated genes in keloid scar tissue, while green dots denote significantly upregulated genes in Dupuytren's disease tissue. Grey data points represent not significantly regulated genes.

3.2. GO functional enrichement analysis

To better undrstand the biological differences of the two conditions, we performed a functional enrichment analysis to determine dysregulated Gene Ontology (GO) categories (Fig. 3) [22,23]. The analysis unveiled a pronounced focus on metabolic processes related to glucan and glycogen within the Dupuytren group. The associated terms encompassed glycogen metabolic process (GO:0005977), cellular glucan metabolic process (GO:0006073) and glucan metabolic process (GO:0044042). Moreover, numerous functional biological processes relevant to tendon development such as tendon formation (GO:0035992), tendon cell differentiation (GO:0035990) and tendon development (GO:0035989) exhibited a pronounced concentration among patients affected by Dupuytren's disease. Notably, in keloid samples, processes involved in skin development (GO:0043588) and especially the epidermis (GO:0008544), as well as the differentiation of keratinocytes (GO:0030216) and associated epidermal cell differentiation (GO:0009913) were enriched. For a comprehensive list of all enriched GO terms, refer to the supplementary file (Table S3).

3.3. KEGG pathway analysis, GO functional enrichement analysis of selected pathways and construction of the PPI network including module analysis

A KEGG pathway analysis revealed 29 and 21 significantly enriched pathways (Table S4A and B) for keloids and Dupuytren's disease, respectively, including arachidonic acid metabolism, metabolic pathways, transcriptional dysregulation in cancer and cell adhesion molecules. The Top 5 KEGG pathways are shown in Table 1. In light of the considerable number of enriched genes within the KEGG pathway (hsa01100), we conducted a GO enrichment analysis using the DAVID online tool, employing default parameters based on annotated genes. The objective was to pinpoint pertinent biological processes. Notably, the analysis yielded a robust enrichment of GO terms associated with sphingolipid and eicosanoid metabolic processes. For a comprehensive rundown of these GO terms, please refer to the attached document (Table S5). Significant genes identified via KEGG and GO enrichment analysis were exemplarily visualized in the arachidonic acid metabolism (hsa00590) and the sphingolipid metabolism (hsa00600) pathway using the KEGG pathways maps online tool (Fig. S1).

For further analysis the list of significant DEGs was mapped using the STRING database to construct a PPI network. The Top 10 genes with the highest degrees were identified using the software Cytoscape, assuming that these play a major role in the pathogenesis (Table 2). The MCODE plug-in identified six modules with a score of >5 and more than 6 nodes. Fig. 4 illustrates the PPI network for Module2. The remaining networks can be accessed in supplementary files (Figs. S2–4).

For example, KEGG enrichment analysis of each module showed that the genes in Module 3 (including ALOX15, CYP2C9, PLA2G2F, PLA2G3, PLB1 ITGA3, COL4A3 and ITGA8) were associated with arachidonic acid metabolism, linoleic acid metabolism and ECM-receptor interaction. Table 3 provides a compilation of the Top 3 KEGG pathways associated with each module, ranked by their respective p-values. For Module 1 there were no annotated pathways. A list of all annotated KEGG pathways, which were associated with each individual module can be found in the attachment (Tables S6A–F).

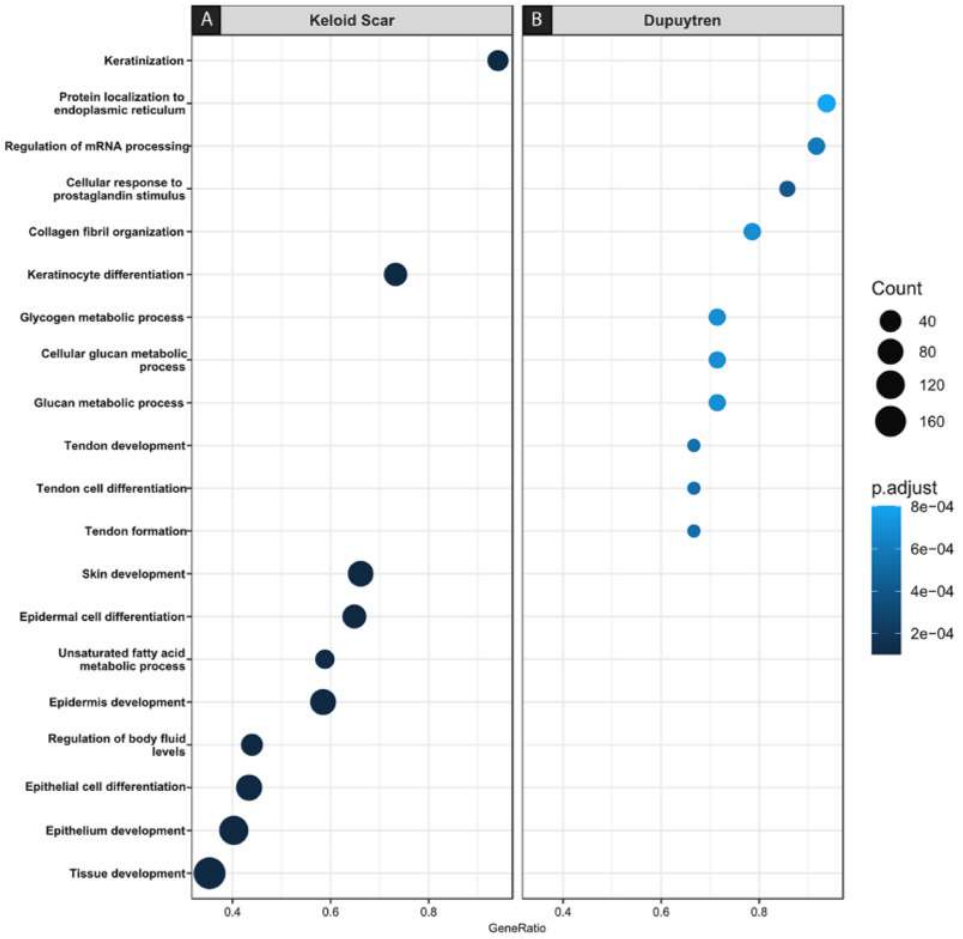

Fig. 3. Dot plot showing the Top 10 enriched GO biological processes of differentially expressed genes in keloid scars (A) and Dupuytren's disease (B). The processes are ranked according to the gene ratio. The Gene ratio is the proportion of genes that significantly correlated with the total number of genes associated to that process. The p-adjusted value is represented by the shading of the dots. The diameter of the dots represents the process specific gene count, which refers to the number of genes associated with each GO biological process.

Table 1
This table displays the KEGG pathway enrichment analysis of the DEGs of keloid scars and Dupuytren's disease samples using the Database for Annotation Visualization and Integrated Discovery (DAVID) online tool. The following cut-off criteria were applied: p-value <0.05 and a gene count ≥ 2.

KEGG pathway term	Description	No. of enriched genes	p-value
Upregulated in keloids			
hsa01100	Metabolic pathways	150	1.37×10^{-6}
hsa00590	Arachidonic acid metabolism	17	1.98×10^{-6}
hsa04530	Tight junction	25	4.51×10^{-4}
hsa00591	Linoleic acid metabolism	9	6.76×10^{-4}
hsa00600	Sphingolipid metabolism	12	7.32×10^{-4}
Upregulated in Dupuytren's samples			
hsa05171	Coronavirus disease - COVID-19	16	1.60×10^{-3}
hsa03010	Ribosome	13	1.85×10^{-3}
hsa05202	Transcriptional misregulation in cancer	14	2.25×10^{-3}
hsa04514	Cell adhesion molecules	12	3.61×10^{-3}
hsa04350	TGF-β signaling pathway	9	4.19×10^{-3}

Table 2
The Top 10 genes selected by the highest degree as calculated using Cytoscape.

Gene symbol	Gene name	Degree	Upregulated in
CDH1	Cadherin 1	332	Keloid
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2	258	Keloid
EGF	Epidermal Growth Factor	256	Keloid
NOTCH1	Notch Receptor 1	200	Keloid
CASP3	Caspase 3	194	Dupuytren's disease
RPS27A	Ribosomal Protein S27a	192	Dupuytren's disease
CXCL8	C-X-C Motif Chemokine Ligand 8	180	Dupuytren's disease
IL10	Interleukin 10	180	Dupuytren's disease
SOX9	SRY-Box Transcription Factor 9	174	Keloid
ITGB1	Integrin Subunit Beta 1	174	Dupuytren's disease

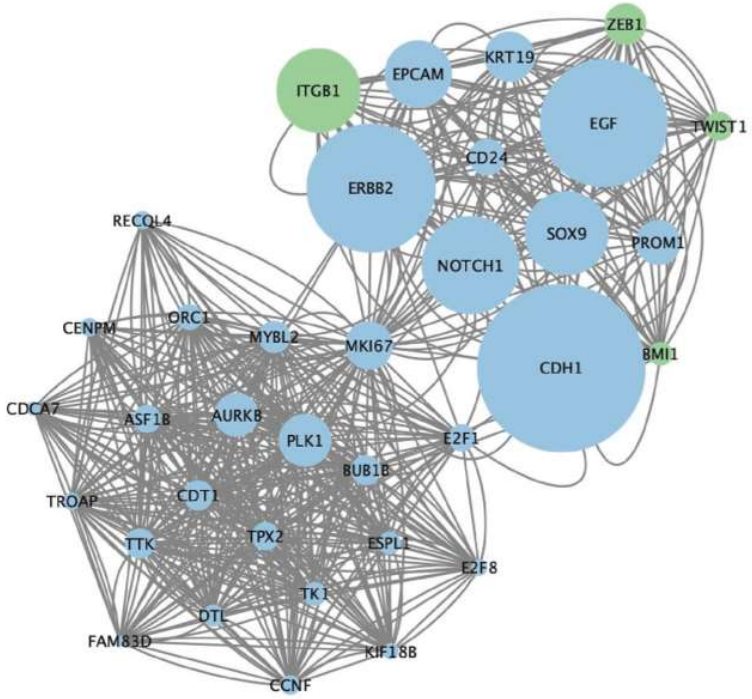

Fig. 4. Significant Modules identified with the MCODE plugin, Module 2 (MCODE score = 16.71, nodes = 35). Blue nodes represent significantly upregulated DEGs in keloids and green nodes represent DEGs within Dupuytren's samples respectively. The node diameter is proportional to the number of degrees of each node.

4. Discussion

Keloid scars and Dupuytren's disease share several clinical and microscopic similarities. For example, both conditions show an accumulation in different ethnic groups. Keloids occur in all races with a preponderance in Africans or people of African descent [24]. By contrast Dupuytren's disease is rare in Africans and mostly affects people of Caucasian descent [25]. Studies have shown that individuals with a positive family history are more susceptible to the disease, and they are more prone to developing an earlier onset coupled with a higher severity. These findings demonstrate a genetic predisposition in both conditions [3,26,27]. The diseases occur in different regions of the body. Whereas Dupuytren's disease mainly affects the aponeurosis of the palms of the hands and rarely the soles of the feet (which is then called Ledderhose's disease), keloids tend to occur within the skin of the chest, shoulders, chin, neck, lower legs and ears [4,5]. Although on a macroscopical level the affected regions appear to be quite distinct, microscopically both share common characteristics. Both keloid scars and Dupuytren's disease seem to form preferentially on mechanically stressed regions [5, 28]. Moreover the conditions are characterized by hyperproliferation of extracellular matrix, particularly collagen type I and III [29, 30]. On a molecular level, in both conditions transforming growth factor beta (TGF-β) has been extensively studied and seems to play a

Table 3
This table shows the top 3 KEGG pathways of significant modules identified using MCODE in Cystoscape and the DAVID online tool.

KEGG pathway term	Description	p-value	Genes
Module 2			
hsa04110	Cell cycle	8.02×10^{-6}	ESPL1, ORC1, PLK1, E2F1, BUB1B, TTK
hsa05219	Bladder cancer	1.06×10^{-4}	CDH1, EGF, ERBB2, E2F1
hsa05215	Prostate cancer	1.40×10^{-3}	ZEB1, EGF, ERBB2, E2F1
Module 3			
hsa00590	Arachidonic acid metabolism	4.73×10^{-21}	CYP2J2, PLA2G2F, PLA2G4F, PLA2G4D, PLA2G4E, PLA2G2A, CYP4F3, ALOX15, PLA2G3, ALOX12, ALOX12B, CYP4F8, ALOX15B, PLB1, PTGS1, CYP2C9
hsa00591	Linoleic acid metabolism	8.54×10^{-14}	CYP2J2, PLA2G2F, CYP2C9, PLA2G4F, PLA2G4D, PLA2G4E, ALOX15, PLA2G2A, PLA2G3, PLB1
hsa04512	ECM-receptor interaction	1.07×10^{-10}	COL2A1, ITGA3, ITGB4, COL4A3, ITGA8, COL4A6, COL4A5, COL9A3, ITGAV, ITGB7, ITGB6
Module 4			
hsa00600	Sphingolipid metabolism	3.60×10^{-11}	CERS3, SMPD3, SMPD2, CERS4, UGT8, ACER1, DEGS2, PLPP2, SGPP2
hsa04916	Melanogenesis	6.20×10^{-5}	DCT, TYRP1, WNT7A, MITF, TYR, WNT4
hsa04071	Sphingolipid signaling pathway	1.35×10^{-4}	CERS3, SMPD2, CERS4, ACER1, DEGS2, SGPP2
Module 5			
hsa05224	Breast cancer	2.13×10^{-6}	FGF5, NOTCH3, FGF16, WNT10A, WNT3A, WNT16, DLL1, FGF22, FGF10
hsa05200	Pathways in cancer	4.82×10^{-5}	NTRK1, NOTCH3, WNT10A, WNT3A, PTCH1, TGFA, WNT16, DLL1, FGF5, FGF16, FGFR3, FGF22, FGF10
hsa05226	Gastric cancer	2.38×10^{-4}	FGF5, FGF16, WNT10A, WNT3A, WNT16, FGF22, FGF10
Module 6			
hsa04151	PI3K-Akt signaling pathway	2.95×10^{-8}	GNG10, CHRM1, GNG2, ERBB3, LPAR5, PDGFD, PDGFC, LPAR1, LPAR2, LPAR3
hsa04072	Phospholipase D signaling pathway	1.69×10^{-5}	LPAR5, PDGFD, PDGFC, LPAR1, LPAR2, LPAR3
hsa04015	Rap1 signaling pathway	9.09×10^{-5}	LPAR5, PDGFD, PDGFC, LPAR1, LPAR2, LPAR3

major role in fibroblast proliferation [31,32]. Regardless of the ever-increasing scientific understanding, both diseases tend to have high recurrence rates, and no definitive cure for either condition has been found. In both cases, surgery remains the mainstay of treatment if joint contractures or aesthetic disturbances occur.

To identify potential molecular targets for therapy and to substantiate the known similarities on a molecular level, we have compared gene expression profiles of the two common fibrotic spectrum disorders. Furthermore, we evaluated the PPI networks and associated pathways, which may play a role in the onset and progression of both diseases.

Our findings suggest characteristic molecular alterations within the two conditions. Interestingly, we found absolutely no overlapping gene expression profiles between them. Our analysis unveiled key biological processes and signaling pathways that could potentially hold pivotal roles in driving their onset and progression. Additionally, we identified ten genes for each disease which appear to be of central importance in disease development. These genes may provide useful starting points for the development of new therapeutic agents.

Differential gene expression analysis revealed 1922 DEGs, including 1401 upregulated genes within the keloid tissue samples and only 521 upregulated genes within the Dupuytren's disease samples. Most upregulated genes were found in keloids, and most of them are mainly associated with skin development and related processes. The reason for the significantly lager fraction of upregulated genes compared to downregulated genes might be due to the great number of genes encoding for skin development and keratinization.

Enrichment analysis of the identified DEGs within the keloid samples revealed several significantly enriched KEGG pathways, including arachidonic acid metabolism, metabolic pathways, tight junctions, and linoleic acid metabolism. In line with our findings is that keloids bear higher levels of arachidonic acid when compared to skin of keloid-prone and non-keloid-prone patients [33]. Arachidonic acid has several downstream products including eicosanoids such as leukotrienes, prostanoids of prostaglandins, prostacyclins and thromboxanes. These molecules are believed to be proinflammatory in nature and thereby contributing to the formation of keloid scars [34]. Furthermore, we found an upregulation of lipid and sphingolipid metabolic processes further suggesting that both processes hold a significant role in chronic inflammation, potentially contributing to keloid formation [34,35]. Little is known about lipid metabolism and inflammation in keloids. Among the enriched KEGG pathways within the Dupuytren's cohort were cell adhesion molecules, transcriptional dysregulation in cancer and the TGF-β signaling pathway. The TGF-β signaling pathway plays an important role in keloid and Dupuytren's disease formation [36,37]. TGF-β is a pivotal component in producing the myofibroblast phenotype

which is responsible for aberrant collagen deposition and contraction in Dupuytren's disease and supposedly keloid scars [38–40]. Therefore, monitoring these signaling pathways may aid in the prediction of the progression of these two diseases.

A PPI network analysis of the DEGs revealed that CDH1, ERBB2, EGF, NOTCH1, CASP3, RPS27A, CXL8, IL10, SOX9 and ITGB1 hold key positions in the pathogenesis of Dupuytren's disease and keloid formation. CDH1, which encodes for the protein E-cadherin, had similar expression levels in keloid keratinocytes when compared to normal keratinocytes. The protein levels of E-cadherin are diminished in keloids and are lost in cancer cells undergoing endothelial-mesenchymal transition (EMT), indicating a switch to mesenchymal markers such as N-cadherin [41–43]. The oncogene ERBB2 was identified to be attenuated in keloids and is believed to play a major role in margin migration via neuregulin-1 (NRG1) [44]. EGF increases fibroblast proliferation and motility [45]. To the more, EGF alters TGF-β1 signaling, which is a major pathway in the pathogenesis of keloid formation, leading to accumulation of ECM components [46]. Although our studies indicate an upregulation of EGF signaling, the evidence in the literature is contradictory, with several studies also suggesting a decreased expression of EGF [47]. The highly conserved Notch pathway is essential to the regulation of key cellular processes and functions such as fibroblast cell proliferation and migration. Notch-1, a member of a family comprising four transmembrane receptors (Notch-1 to Notch-4), has been identified to be increasingly expressed in keloids and hypertrophic scar tissue when compared to normal skin [48–50]. Blocking of Notch signaling resulted in decreased scar formation and prevention of tissue fibrosis in an experimental setting [51,52]. Our results are in line with these findings and suggest a crucial role for Notch signaling in the development of keloids. In addition, SOX9, which is important for chondrogenesis, was significantly upregulated [53].

In a previous investigation, Jung et al. identified that terms related to collagen and ECM were enriched in tissue samples of patients suffering from Dupuytren's disease [54]. In the present study DEGs, including ITGB1, COL2A1, ITGA8 and ITGAV, were predominantly enriched in the ECM-receptor interaction pathway. In accordance with that, Layton et al. found that pericytes derived from Dupuytren's disease nodules express several integrin receptors including ITGB1 and ITGAV [55]. It may therefore be speculated that these pathways and genes contribute to the progression of Dupuytren's disease. In addition, a KEGG pathway analysis of each individual module revealed that pathways, which also play a role in the development of different cancerous diseases, appear to be enriched in the fibrotic diseases assessed in our study. Using PCR analysis, Docheva et al. showed that fibronectin-binding integrins β3 and β5 are upregulated in Dupuytren's disease [56].

It has to be kept in mind, however, that the sample size in our study was small and additional experiments, for example reverse transcription-quantitative polymerase chain reaction, were not performed to confirm mRNA expression levels. Thus, further studies are required to verify these genetic signatures.

5. Conclusion

Our study showed that Dupuytren's disease and keloids, despite some obvious similarities, share indubitably no overlapping gene expression profiles. Our results indicate that CDH1, ERBB2, EGF, NOTCH1 and SOX9 play a crucial role in the onset and development of keloids. On the other hand, CASP3, RPS27A, CXCL8, IL-10 and ITGB1 play a pivotal role in the pathogenesis of Dupuytren's disease.

We identified important upregulated KEGG pathways of both keloids and Dupuytren's disease, some of which haven't been described in the literature before and therefore warrant further investigation. They include metabolic pathways (hsa01100), arachidonic acid metabolism (hsa00590) and transcriptional dysregulation in cancer (hsa05202).

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Data availability statement

Data associated with our study has not been deposited into a publicly available repository. Data included in the article/supp. Material/referenced in the article will be made available upon request.

Declarations of Interest: none.

Ethics declarations

- This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the LMU Medical Faculty (AZ 19–177).
- All participants/patients (or their proxies/legal guardians) provided informed consent to participate in the study.
- All participants/patients (or their proxies/legal guardians) provided informed consent for the publication of their anonymised case details and images.

CRediT authorship contribution statement

Marcus Stocks: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Software. **Annika S. Walter:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. **Elif Akova:** Software, Visualization, Formal analysis. **Gerd Gauglitz:** Writing – review & editing. **Attila Aszodi:** Project administration, Resources, Supervision, Writing – review & editing. **Wolfgang Boecker:** Resources.

Maximilian M. Saller: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Elias Volkmer:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A

The appendix is an optional section that can contain details and data supplemental to the main text—for example, explanations of experimental details that would disrupt the flow of the main text but nonetheless remain crucial to understanding and reproducing the research shown; figures of replicates for experiments of which representative data is shown in the main text can be added here if brief, or as Supplementary data. Mathematical proofs of results not central to the paper can be added as an appendix.

Appendix B

Figure S1 to Figure S4
Table S1.
Table S2.
Table S3.
Table S4 A and B.
Table S5.
Table S6A to Table S6F

Appendix C. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23681.

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5 Summary

Wound healing and scar formation is a complex biological process that takes place countless times during life. The regeneration of tissue is the ability of the human body to adapt to the environment and to damage, such as injuries. The human skin is the biggest organ with the greatest exposure to potentially harmful environmental influences.[5] Depending on causes and individual risk factors, the tissue may not be able to recover and restore its original condition.[19,23,25] Wound healing disorders and pathological scar formations are widespread in human beings and a challenge for health care systems.[35,36,40]

Due to its histological and clinical characteristics, scar tissue develops within the framework of the fibrotic remodelling processes in the connective tissue.[48,49] Fibrotic processes can affect different tissues and organs in the body. This project includes skin scar formation with normal skin scars, hypertrophic skin scars and keloid skin scars as well as Dupuytren´s scar tissue. As a thematic introduction to hypertrophic scar formation, we conducted a systematic review about molecular interactions during burn wound healing and their assignment to wound healing phases (Article III, Attachment A).[38] Our visual created molecular network was published online as an interactive illustration with open-free access.

In this research project, fresh scar tissue samples and Dupuytren´s disease tissue samples were collected during hand surgeries on site in the operating room. In the experimental part, RNA was isolated followed by library preparation and next-generation sequencing with subsequent bioinformatical procession and evaluation. We hypothesized that normal scars, hypertrophic scars and keloid scars show different gene expression profiles in Bulk-RNA sequencing (Article I).[1] Our results demonstrate that normal skin scars and hypertrophic skin scars show an overlapping clustering in LDA-Analysis and a common expression of 8 upregulated genes as well as 9 downregulated genes in Venn diagram. Keloid scars represent an independent group without clustering with normal or hypertrophic scars. There are no shared expressed genes in keloid scars and hypertrophic scars.

In Summary, normal scars and hypertrophic scars are much more similar to each other on a transcriptional level than to keloid scars. This leads us to conclude that keloid scars do not evolve from hypertrophic scars. Despite different gene expression profiles of hypertrophic scars and keloid scars, the guidelines for the treatment of pathological scars of the German Society for Dermatology mention similar therapeutic pathways for both types of scars.[63] There exist a variety of therapeutic options for pathological scars, including conservative topical applications, surgical corrections, laser therapy, cryosurgery, compression treatments, microneedling and infiltration treatments.[63] The success of these therapies depends, at least in part, on the underlying pathological scar type. Importantly, several clinical and case studies describe an increased risk of recurrence of keloid scars after surgical

excision, compared to hypertrophic scars.[45,64] Differences in gene expression profiles are a possible explanation for different rates of success with regards to the therapy of those scars.

In the second part of this research project, gene expression profiles of keloid scars and Dupuytren´s disease tissue were compared (Article II).[2] Although they show several clinical and microscopic similarities, there is no shared significant gene expression or overlapping clustering in principal component analysis or Venn diagram. After we identified the top 10 expressed genes sorted by type of tissue which can be important for development of pathogenesis. We performed a protein-protein network and visualized protein interactions using Cytoscape. These results, with the identification of potentially pathogenic important genes, may influence further research of treatment options for affected patients.

It may be possible to extend our project to other fibroproliferative disorders in future research projects with a larger sample size of pathological scars and other types of fibrotic tissue. Additionally, a future research approach could focus on technical improvements of RNA isolation from fibrotic tissues. RNA isolation of fibrotic tissues poses a challenge due to their increased amount of connective tissue and their decreased cell counts. Finally, our bioinformatical analysis is based on results from Bulk-RNA sequencing. It would be particularly interesting to use Single-Cell-RNA sequencing to obtain detailed information about cell types in fibrotic tissue and their gene expression profiles. In summary, this research project highlights multiple new starting points for further fundamental biomolecular research as well as the development of new therapeutic options.

6 Zusammenfassung

Die Wundheilung und Narbenbildung ist ein komplexer biologischer Prozess, der im Laufe des Lebens unzählige Male abläuft. Die Regeneration von Gewebe ist eine besondere Fähigkeit des menschlichen Körpers, sich an Umwelteinflüsse und äußerliche Verletzungen anzupassen. Die Haut ist das größte Organ des menschlichen Körpers und durch den direkten Kontakt zur Umwelt, häufig schädlichen Umwelteinflüssen ausgesetzt.[5] Je nach Ursachen und individuellen Risikofaktoren ist das Gewebe nicht in der Lage, sich zu erholen und den ursprünglichen Zustand wiederherzustellen.[19,23,25] Wundheilungsstörungen und pathologische Narbenbildungen sind in der Bevölkerung weit verbreitet und stellen durch ihre Tendenz zu Chronifizierungen und ungünstigen Verläufen eine Herausforderung für das Gesundheitssystem dar.[48,49] Aufgrund ihrer histologischen und klinischen Merkmale entsteht Narbengewebe im Rahmen eines fibrotischen Umbauprozesses des Bindegewebes. Fibrotische Prozesse können verschiedene Gewebe und Organe im Körper betreffen. Dieses Forschungsprojekt befasst sich mit der Narbenbildung der Haut, insbesondere mit physiologisch unauffälligen Hautnarben, hypertrophen Hautnarben und Keloiden der Hand sowie Gewebe von Morbus Dupuytren. Als thematischen Einstieg in die hypertrophe Narbenbildung haben wir eine umfangreiches Systematic Review über molekulare Interaktionen bei Wundheilung nach Verbrennungsverletzungen mit Zuordnung von transkriptionellen Prozessen zu Wundheilungsphasen erstellt (Artikel III, Anhang A).[38] Unser visuell erstelltes molekulares Netzwerk wurde online als interaktive Illustration mit kostenfreiem, öffentlichem Zugang veröffentlicht.

In diesem Forschungsprojekt wurden mehrere frische Gewebestücke von Narbengeweben und Morbus Dupuytren-Gewebeproben während einer handchirurgischen Operation vor Ort im Operationssaal entnommen und verarbeitet. Im experimentellen Teil wurde RNA isoliert und verarbeitet, sowie die Daten anschließend bioinformatisch verarbeitet und analysiert. Grundlage der Forschungsarbeit war die Hypothese, dass normale Narben, hypertrophe Narben und Keloide bei der Bulk-RNA Sequenzierung unterschiedliche Genexpressionsprofile aufweisen würden (Artikel I).[1] Unsere Ergebnisse zeigen, dass normale Hautnarben und hypertrophe Hautnarben in der LDA-Analyse ein überlappendes Clustering und eine gemeinsame Expression von 8 hochregulierten Genen und 9 herunterregulierten Genen im Venn-Diagramm aufweisen. Keloide stellen eine unabhängige Gruppe dar, die nicht mit normalen oder hypertrophen Narben auf Basis ihrer Genexpression clustern. Es gibt keine gemeinsam exprimierten Gene in Keloiden und hypertrophen Narben. Zusammenfassend lässt sich sagen, dass normale Narben und hypertrophe Narben einander auf der Transkriptionsebene ähnlicher sind als Keloide. Basierend auf diesen wissenschaftlichen

Erkenntnissen über die Genexpressionsprofile dieser pathologischen Narbentypen, entwickeln sich Keloide nicht aus hypertrophen Narben.

In den Leitlinien zur Behandlung pathologischer Narben der Deutschen Gesellschaft für Dermatologie[63] werden für hypertrophen Narben und Keloide ähnliche Therapiepfade genannt, trotz unterschiedlicher molekularbiologischer Genexpressionsprofilen. Es werden eine Vielzahl von Therapieoptionen für pathologische Narben genannt, darunter konservative topische Anwendungen, chirurgische Korrekturen, Lasertherapie, Kryochirurgie, Kompressionsbehandlungen, Microneedling und Infiltrationsbehandlungen.[63] Der Therapieerfolg der einzelnen Behandlungsoptionen hängt zudem vom zugrunde liegenden pathologischen Narbentyp ab. Mehrere klinische Studien und Fallstudien beschreiben ein erhöhtes Rezidivrisiko von Keloiden nach chirurgischer Exzision im Vergleich zu hypertrophen Narben.[45,64] Unterschiede in den Genexpressionsprofilen werden als mögliche Erklärung für die unterschiedlichen Therapieerfolge von hypertrophen Narben und Keloiden diskutiert. Diese Erkenntnisse bilden die Grundlage für die Entwicklung zukünftiger klinischer Behandlungsoptionen auf der Basis von Genexpressionsprofilen einzelner Narbentypen.

Im zweiten Teil dieses Forschungsprojekts wurden die Genexpressionsprofile von Keloiden und Morbus Dupuytren-Gewebe verglichen (Artikel II).[2] Obwohl sie mehrere klinische und mikroskopische Ähnlichkeiten aufweisen, gibt es keine gemeinsame signifikante Genexpression oder überlappende Clusterbildung in der Hauptkomponentenanalyse oder im Venn-Diagramm. Nach der Identifizierung der Top 10 am stärksten exprimierten Gene, sortiert nach Gewebetyp, die für die Entwicklung der Pathogenese wichtig sein können, wurde ein Protein-Protein-Interaktionsnetzwerk erstellt, das mit Cytoscape visualisiert wurde. Diese Ergebnisse mit Identifizierung potenzieller pathogener molekularer Schlüsselstellen, bilden die Grundlage für zukünftige Forschungsprojekte mit Schwerpunkt auf Behandlungsmöglichkeiten pathologischer Narbenbildungen und fibrotischer Erkrankungen für betroffene Patienten.

In zukünftigen Studien wären weitere Forschungsprojekte mit einer größeren Stichprobenanzahl von Narben und anderen fibrotischen Geweben denkbar, um unser Projekt auf weitere fibroproliferative Erkrankungen auszuweiten. Zusätzlich könnten technische Verbesserungen der RNA-Gewinnung aus fibrotischen Geweben ein zukünftiger Forschungsansatz darstellen. Fibrotische Gewebe stellen aufgrund ihres hohen Anteils an Bindegewebe und ihrer geringeren Zellzahl eine technische Herausforderung dar. Unsere bioinformatische Auswertung basiert auf den Ergebnissen der Bulk-RNA Sequenzierung. Daher wäre es besonders interessant, die Single-Cell-RNA Sequenzierung einzusetzen, um detaillierte Informationen über Zelltypen in fibrotischem Gewebe und deren Genexpressionsprofile zu erhalten. Zusammenfassend lässt sich sagen, dass dieses Forschungsprojekt zahlreiche

neue Ansatzpunkte für die weitere biomolekulare Grundlagenforschung sowie für die Entwicklung neuer therapeutischer Optionen bietet.

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8 List of figures

9 Supplementary data

9.1 Attachments to Article I

Appendix https://drive.google.com/drive/mobile/folders/1dII7UjZUnpXfKhpp4S4sRNdJxTTOc1E6?usp=sharing	Table S1 Table S2A_C Figure S1 Figure S2

9.2 Attachments to Article II

Appendix A https://docs.google.com/spreadsheets/d/15GCGpj8ZWGeakxWPHa8zpc3zojO22sji/htmlview	Table S1 Table S2 Table S3 Table S4 A and B Table S5 Table S6A to Table S6F
Appendix B https://free.qr1.at/l/media?f=fa25ebb6f01d56048f08a3ad886be85c&sid=9073402	Figure S1 Figure S2 Figure S3 Figure S4

9.3 Article III: Systematic review of molecular pathways in burn wound healing

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Review

Systematic review of molecular pathways in burn wound healing

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ABSTRACT

Depending on extent and depth, burn injuries and resulting scars may be challenging and expensive to treat and above all heavily impact the patients' lives. This systematic review represents the current state of knowledge on molecular pathways activated during burn wound healing. All currently known molecular information about gene expression and molecular interactions in mammals has been summarized. An ample interaction of regenerative cytokines, growth factors, ECM-regenerative molecules and proinflammatory immune response became apparent. We identified three molecules to be most often involved in the pathways: TGFB1, ACTA1 and COL1A1. Yet, other factors including FLII, AKT1 and miR-145 were shown to play pivotal roles in burn wound healing as well. This systematic review helps to explain the fundamental molecular proceedings participating in burn wound healing. A number of new molecular interactions and functional connections were identified yielding intriguing new research targets. An interactive version of the first network about molecular pathways and interactions during burn wound healing is provided in the online edition and on WikiPathways.

Contents

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1. Introduction

Healing of burn wounds is a complex spatiotemporal process which is accompanied by acute and long-term consequences including continuing psychological and physiological problems for affected patients. Especially patients with high degree burn wounds need intensive care in special burn trauma centers as well as extensive surgery. This may even include multiple skin transplants. After months of acute and rehabilitative care, these patients often suffer from pathological scar formations, such as hypertrophic scars or atrophic contractions, which mostly result in some degree of impaired mobility as well as aesthetic claims [1,2].

Burn injured casualties are patients with a very high need for intensive medical care. In Germany, 1088 adult and 2129 adolescent burn patients were officially registered in burn care centers in the year 2019 [3]. In 2019, 39 German hospitals specializing in burns were part of the official burn register of the German Association of Burn Treatment. The number of registered burn patients treated in these special burn centers decreased over the years. On average, 13.9% of the body surface area was affected in adults with burn wounds. Data of the German Association of Burn Treatment shows that the intensive care for burn patients costs around 4600 € per percent of the burned total body surface area (TBSA). Thus, the cost for acute and life-saving treatment of burn patients costs 64,000 € per case on average. However, the costs of rehabilitation are significantly higher and long-term care phases after acute hospitalization phases can take several months up to years [4].

Burn wounds differ in type, depending on the temperature and the duration of the exposure to the heat source. The pressure applied by the heat source as well as its aggregate state also influence the type of burn wound [1,5]. Pathophysiologically, it is known that heat of 69 °C for one second on the epidermis is enough to produce necrotic skin processes [6]. Necrosis induces a general inflammatory reaction in the affected tissues. The inflammatory process along with the necrosis damage skin tissue, including small capillaries, which are essential for nutrition and oxygen supply of the skin [7]. This thermally induced destruction enables fluids to pass the damaged endothelial cells of skin capillaries and starts a release of proinflammatory cytokines [8–10]. This ultimately results in the formation of local oedema in the burned area and induces systemic reactions such as hypovolaemia-associated shock symptoms, including potential life-threatening cardiac complications [9,11]. In order to prevent massive oedema and shock symptoms, protein-rich fluid substitution is necessary [6,7].

Wound healing consists of complex molecular mechanisms and interactions and can be delineated into three distinct wound healing phases independent of the type of skin injury: Inflammatory phase, proliferation phase and remodeling phase [12]. The time span for wound healing in humans after an acute trauma is mentioned in the literature at 48–72 h for the inflammation phase, 5–10 days for the proliferation phase and about 3 weeks for the remodeling phase [13,14]. Each wound healing phase shows a smooth, individual transition, which makes it difficult to specifically distinguish the different phases. There are many different types of wounds such as sharp cuts, blunt crush wounds and burn wounds. On the one hand, all types hurt skin and tissue, cause bleedings and initiate the inflammation phase [13]. On the other hand, burn wounds differ from cut wounds especially in the extent of the affected area as well as varying degrees of severity. In particular, third-degree burns with extensive affected areas and penetration of multiple skin layers correlate with the risk of a systemic inflammatory reaction which can cause a severe loss of fluids in particular. Hypovolemia and burn shock are some of the most important factors specifically caused by with widespread third-degree burns. Especially the increased capillary permeability and decreased interstitial fluid pressure lead to subsequent detrimental therapeutic consequences in patients with burn wounding [13,15]. Burn wound healing is even more individual and therefore more difficult to differentiate the individual phases. Even if wound healing follows this general pattern, each patient with deep burn wounds differs to another. Individual factors like age, sex, pre-existing conditions, type of accident, depth of wound, temperature of applied

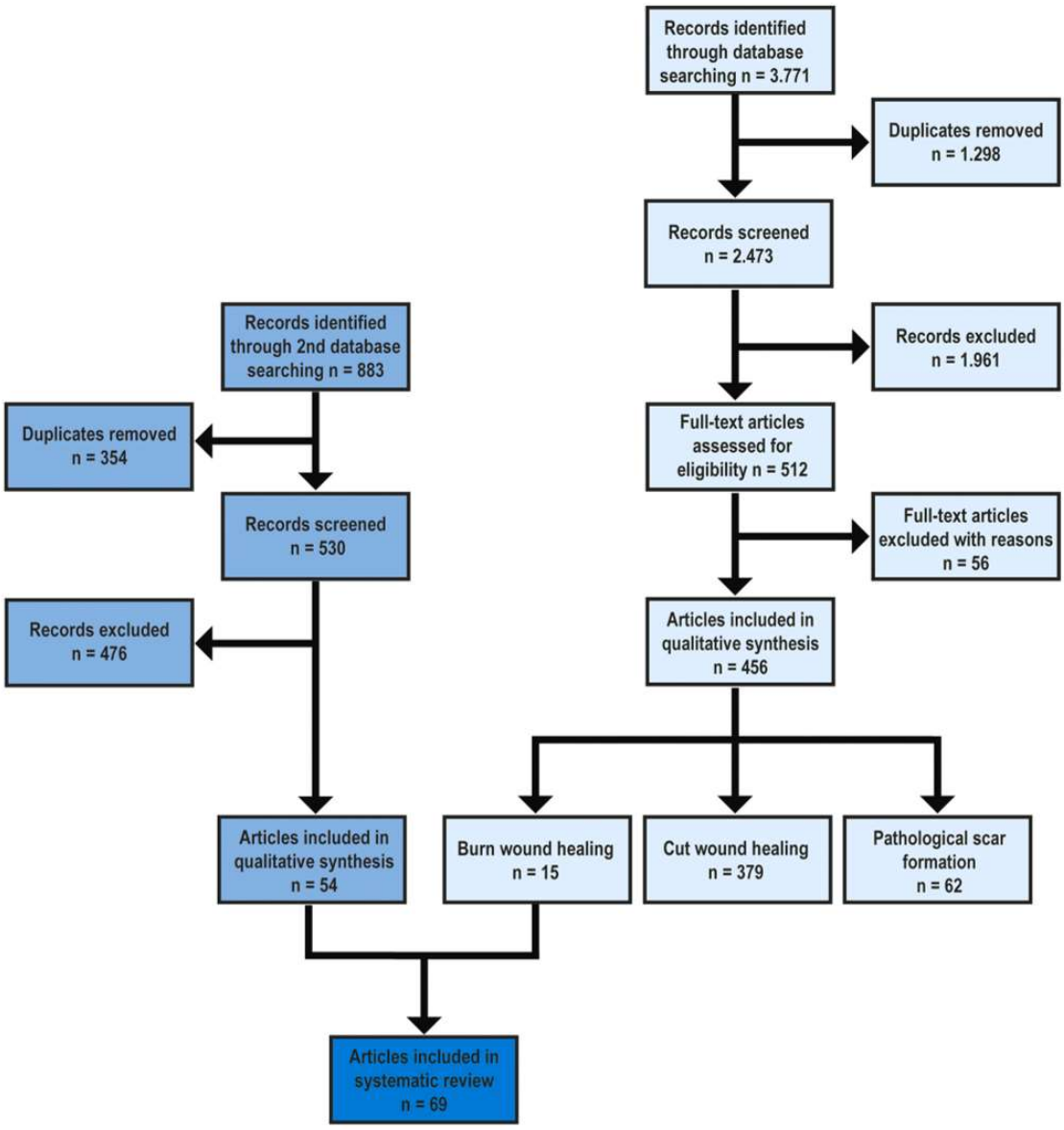

Fig. 1 – : *Flow-chart of systematic literature search about burn wound healing.* Following the PRISMA guidelines, after searching with all key word combinations, we counted 3771 articles in the first search round (right arm). After eliminating duplicates and screening the remaining articles, we excluded articles according to the exclusion criteria. The included articles were separated into three main categories: burn wound healing, cut wound healing, pathological scar formation. Despite the high rate of new publications about wound healing, we only gathered 15 articles matching our inclusion criteria for the systematic review. We thus performed a second check with more detailed key words focused on thermal injuries (left arm). Both search procedures followed the same inclusion and exclusion criteria. In the end, 69 articles were included in this systematic review.

heat, wound infection etc. make it difficult to determine a fixed time frame for individual wound healing phases. Wound Healing in humans is different to wound healing in an experimental setting with mice or rats [16]. The environment, rate of cell proliferation, skin contractions and structure of skin appendages differs compared to wound healing in humans [16]. In addition, it is technically challenging to represent time factors visually. The detailed molecular mechanism of wound healing, especially after thermal injuries, is currently not well understood.

The profibrotic, potent cytokine transforming growth factor 1 (TGFB1) leads to an upregulation of many molecular factors like alpha smooth muscle actin (ACTA1) [17], heat shock protein 47 (SERPINH1) [18] or collagen type 1 (COL1A1) [18]. ACTA1 positive myofibroblasts can stimulate re-epithelization, matrix deposition and are involved in contraction during wound closure and scar formation [19,20].

In contrast, interferon alpha 2 (IFNA2) has antifibrotic properties by decreasing the production of fibronectin and different collagens during wound healing [21–23]. Various molecular factors and different types of interactions are involved in the regulation of wound healing. The correct balance between spatiotemporal stimulation and suppression of specific pathways is pivotal in obtaining optimal wound healing results. Up to now, no systemic summary of molecular biological influences on burn wound healing has been available. The aim of this systematic literature review was to collect all involved molecular players and to visualize the essential pathways in burn wound healing. It serves as a starting point for a systematical, biological view of molecular interactions in burn wound healing to enable prospective new research approaches. The major molecular key players for burn wound healing are discussed.

2. Methods

This systematic literature review was registered in December 2019 on the International prospective register of systematic reviews PROSPERO (Registration-ID: CRD42019150478) and follows the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) protocol. Two reviewers per screening phase worked independently of each other to avoid bias. To maintain equivocality, discrepancies in individual studies were discussed with specialists.

2.1. Search strategy

We started our general database PubMed search with the following keywords and their combinations: ("myofibroblast" OR "myofibroblasts" OR "epithelial cell" OR "epithelial cells" OR "keratin cell" OR "keratin cells" OR "fat cell" OR "fat cells" OR "fibroblast" OR "fibroblasts") AND ("skin") AND ("defect" OR "lesion" OR "injury" OR "healing" OR "scar" OR "contraction" OR "mechanical tension") AND ("lesion" OR "injury" OR "epithelial cell" OR "scar AND wound" OR "contractile" OR "mechanotransduction"). After getting an overview of the current research point to the topic "wound healing", more detailed and burn associated wound healing articles were necessary. In order to specify the literature search on burn wound healing, a second database PubMed search with more specific molecular burn wound healing associated key words and combinations was conducted: ("burn wound" OR "burn wounds" OR "burns" OR "thermal injury") AND ("skin") AND ("scar" OR "healing") AND ("gene" OR "expression" OR "pathway" OR "molecular biology" OR "DNA" OR "miR" OR "RNA" OR "protein"). In both search sections special signs "* " as well as "#" were used in our literature search in order to receive all applicable articles and possible MESH terms. A complete summary of all used key words and key word combinations as well as the number of articles found on PubMed can be viewed in the attachment (Supplementary Table 1). The number of articles received with these keyword combinations was recorded and evaluated (Fig. 1).

2.2. Inclusion and exclusion criteria

After downloading the obtained publication lists to a reference manager (Mendeley, Elsevier, USA), duplicates were removed automatically and residual manuscripts were screened for inclusion and exclusion criteria via title and abstract. As inclusion criteria, we determined English language, experimental studies (in vivo and in vitro from mammals, including humans) that included physiological molecular pathways. We excluded non-English language, review articles, case reports, animals other than mammals, synthetic grafts, skin diseases, tissue engineering studies, genetic animal studies without control group, extrinsic treatment or interventional studies, pathological skin diseases and clinical studies. Due to the missing histological analysis of the same patients over a longer time period, clinical studies were excluded. This review offers a qualitative description of burn wound healing as a snapshot to one point in time (April 2022) and enables participation of others in the design process thanks to its free access.

2.3. Data extraction

Initial data extraction started after all articles relevant for the molecular pathways had been analyzed with the data being collected in tables. Information about humans or type of animal, in vivo or in vitro studies, cell types, genes, proteins, type of injury and PubMed-ID were collected. Each extraction of information was checked by team members working independently.

2.4. Data analysis and quality appraisal

All qualitative data was transferred to PathVisio (Version 3.3.0, Department of Bioinformatics, Maastricht University, Netherlands) for the creation of molecular interactions and pathways separated by species and published on WikiPathways (Homo sapiens, Mus musculus, Rattus norvegicus, Sus scrofa domesticus). A merged pathway with all included single species separated pathways created via Cytoscape can be seen in Fig. 2. Depending on the number of mentions and of interactions, the round protein symbols were colored from light- to dark-colored. Supplementary table 2 represents all descriptive genes which have no experimentally validated regulatory function. It might be

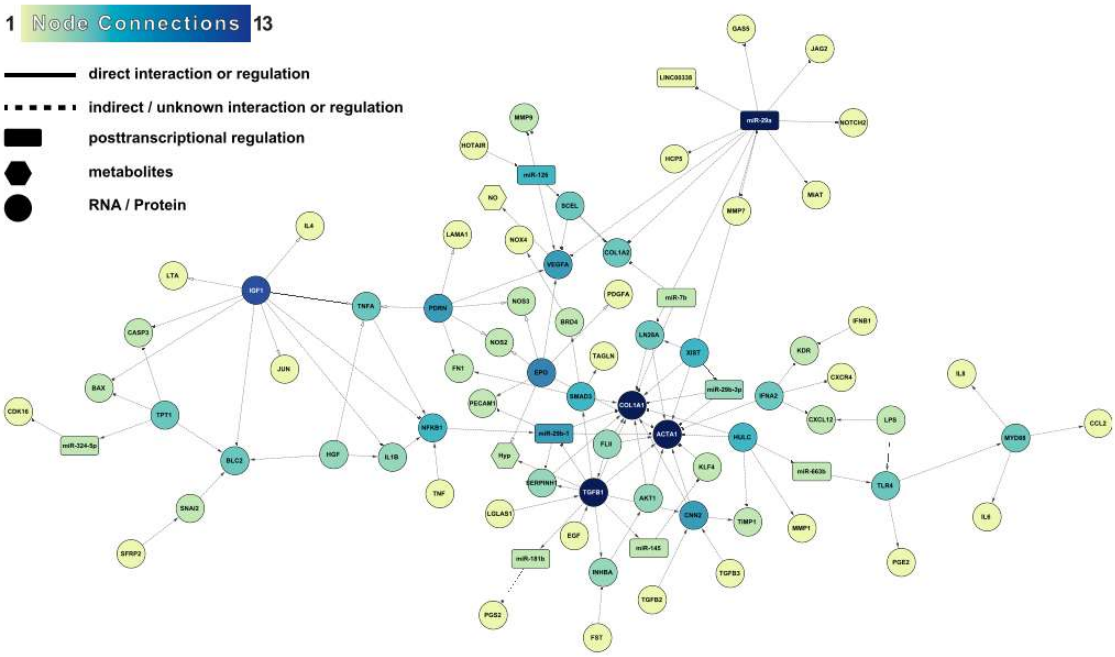

Fig. 2 – : *Network formation via Cytoscape.* This network includes and merges all created pathways, designed in PathVisio, of all included species. It shows new molecular interactions and gives an overview of the current state of knowledge on molecular proceedings during burn wound healing. Colour represents the frequency of their occurrence during the merge of all pathways. Round circles symbolize proteins. Post-transcriptional regulations are visualized as rectangular symbols. Metabolites like Nitric oxide and Hydroxyproline are marked as hexagonal symbols. Solid lines: direct regulations; dashed lines: indirect regulations; arrowheads: activation, t-bars: inhibition.

possible that these genes play a part in similar pathways, but there is no specific experimental evidence for involvement in burn wound healing. Thus, all descriptive genes were excluded from the final merged network. For network formation and analysis, the individual species pathways of humans, mice and rats were merged via Cytoscape version 3.8.1 [24].

3. Results

After defining key words and key word combinations, we started the literature search and sorting process. In Fig. 1, our step-by-step process is visualized. After sorting all relevant articles according to our defined inclusion and exclusion criteria, only 15 articles were included in our systematic review about burn wound healing. Thus, a second literature search with new, extended key words was carried out (Fig. 1, left arm). Finally, 61 articles about burn wound healing were included in this systematic review.

After extraction of data, creation of molecular pathways via PathVisio and merging them via Cytoscape, new molecular interactions and clusters were found (Fig. 2). This network represents the current state of molecular research on burn wound healing and shows new potential research points. In the final network, in vivo as well as in vitro studies were included. Of course the data presented here is derived from a systematic review of the literature and not from actual experiments, thus care must be taken for the interpretation of the results. Due to the technical limitations and a lack of content, no visualization of single gene expression to a time line or correlation to the three wound healing phases was possible. An extensive and interactive version of the molecular network is published on WikiPathways and in the online edition. The online edition contains additional information on the temporal expression of the most important genes involved in wound healing.

3.1. *Molecular reviews as an asset for future research*

In the last years, three essential key players for burn wound healing have been identified. *TGFB1*, *ACTA1* and *COL1A1* are the central molecular factors during wound healing. For each molecular key factor numerous single pathways are known, but overlapping interactions are missing. This systematic review focuses on molecular connections between these well-known key factors and their known single pathways to fill in the knowledge gaps in-between and to form a common pathway which uncovers small molecular intricacies. The synthesis of all known pathways offers as opportunity for future research. Single molecular factors such as *Flii*, *Act1* and

miR-145, among others, which earlier appeared to be less important, receive more attention. This review serves as an introduction to the complexity of molecular pathways during burn wound healing and gives the opportunity to create new research hypothesis.

3.2. *Rarely mentioned molecular factors in the center of attention*

3.2.1. *Top named genes in pathway*

Molecular network formation forms the basis of systems biology. Fig. 2 represents the current state of research on gene expression and molecular interactions during burn wound healing. Solid lines show direct stimulation / inhibition which have been verified experimentally. Dotted lines show indirect stimulation / inhibition due to a lack of evidence for direct influence. The top three most investigated nodes are *TGFB1*, *ACTA1* and *COL1A1* (colored dark blue) which make up the key factors. Especially often contemplated genes are *IGF1*, *EPO*, *CNN2*, *PDRN*, *miR-29-b1*, *miR-29a*, *VEGFA*, *SMAD3*, *miR-126* and *XIST* (colored middle blue, dark green). There are also some genes that are mentioned several times like *TNFA*, *NFKB*, *IFNA2*, *BCL2*, *HGF*, *IL1B*, *SERPINH*, *AKT1*, *INHBA*, *FLII*, *COL1A2* and *TLR4 among others* (colored middle green). Following genes belong to the rarest mentioned genes: *JUN*, *BAX*, *TAGLIN*, *EGF*, *TIMP1*, *FST*, *PDGFA*, *CXCR4*, *IFNB1* and *LAMA1* (colored yellow). In this systematic analysis, one group of molecular factors went unnoticed. Light green colored genes are genes which are mentioned twice such as *SNAI2*, *BRD4*, *FN1*, *PECAM1*, *NOS2*, *NOS3*, *KLF4*, *MMP9*, *KDR* and *CXCL12*. *They* are important to finally connect the known single pathways around the top three genes *TGFB1*, *ACTA1* and *COL1A1* and to fill in the gaps.

3.2.2. *Top counted interactions in pathway*

Looking at most interactions, *TGFB1* and *COL1A1* lead with 12 each, followed by *ACTA1* with eleven interactions to differential factors. *IGF1* has nine interaction partners, CNN2, PDRN, *miR-29b-1*, *miR-29a* all have six known molecular interactions during burn wound healing. *XIST* and *VEGFA* built up the network with five connections for each, followed by *IFNA2*, *MYD88*, *SCEL*, *TNFA* and *NFKB1* with four interactions each.

4. Discussion

4.1. *TGFB1 is currently the key growth factor during burn wound healing*

Growth factors and their interactions play an important role during cell proliferation, cell interactions and tissue regeneration.

Flightless I (Flii) is highly expressed in human burn wounds and hypertrophic scars [25]. As a transcriptional coactivator, Flii leads to an increase in the amount of myofibroblasts by upregulation of transforming growth factor β1 (*Tgfb1*) and alpha-smooth-muscle-actin (*Acta1*) [25,26]. These newly differentiated myofibroblasts deposit high levels of the extracellular matrix protein collagen I (Col1a1) [25,27]. Thus, upregulation of *Flii* during burn wound healing drives the (pathological) thickening of the scar and potentially intensifies scar contractions.

Besides Flii, Epidermal growth factor (EGF) also stimulates the expression of TGFB1 [28]. TGFB1 as a proliferating and profibrotic cytokine acts as a key factor for multiple processes during wound healing. It is involved in differential molecular reactions and is necessary for optimal cell interactions. TGFB1 seems to be species independent and thus forms a central molecular player in burn wound healing. Transcription factor SMAD3 (Mothers against decapentaplegic homolog 3) is activated through phosphorylation by TGFB1 [20]. By building complexes with other SMAD-molecules, SMAD3 is able to stimulate the gene expression of bromodomain-containing protein 4 (BRD4), Fibronectin 1 (FN1) and Transgelin (TAGLN) [20,29]. BRD4 stimulates NADPH oxidase 4 (NOX4), which is part of Proto-oncogene tyrosine-protein kinase ROS (ROS) and necessary for controlling the oxygen level in cells [20,30].

Moreover, TGFB1 stimulates the gene expression of Calponin-2 (CNN2)/Connective tissue growth factor (CTGF) in order to activate cell contractions and mechanical wound closure via ACTA1 after skin injuries [31]. CNN2 can also be activated by TGFB2 and TGFB3 [31]. ACTA1 stimulates profibrotic ECM-remodeling and cell contractions e.g. in myofibroblasts [31]. Acta1 is inhibited by Erythropoietin (Epo) [32] which is an extracellular matrix protein. Furthermore Epo stimulates the expression of other growth factors like platelet-derived growth factor subunit A (Pdgfa) [32] and vascular endothelial growth factor A (Vegfa) [32].

4.2. *Regeneratively and effectively remodeling extracellular matrix molecules during burn wound healing*

After a thermal injury, the damaged tissue is regenerated through rebuilding and remodeling of extracellular matrix (ECM). TGFB1 stimulates the expression of collagen alpha 1 (COL1A1) [18,27,33], which is also stimulated by transcription factors SMAD3 [20] or CNN2 [31]. Collagen 1 is essential in building up extracellular matrix and it interacts with several proteins that support profibrotic tissue remodeling. Transcriptional regulation of collagen 1 can be positively modulated by RAC-alpha serine/threonine-protein kinase (AKT1) [34] or miR-126 [35,36]. Interestingly, miR-126 also leads to increased expression of collagen degrading matrix metalloproteinase-9 (MMP9) [35,37].

However, other extracellular matrix molecules like FN1 [20,38], laminin subunit alpha1 (Lama1) [38] and matrix metalloproteinase inhibitor 1 (Timp1) [39] are also embedded into the molecular network. In order to strike a balance between building and dismantling damaged tissue, inhibiting factors such as miR-29a [40], miR-29b-1 [18] and miR-29b-3p [41] are necessary.

4.3. *Inflammatory molecules as immune response during burn wound healing*

Each injury activates multiple inflammatory reactions. Inflammatory molecules will initially release, to degenerate burned tissue areas and to give the capability for tissue regeneration. Tumor necrosis factor alpha (TNF) is a central

wound healing activated cytokine which stimulates the transcriptional expression of nuclear factor NF-kappa-B p105 subunit (NFKB1) [18] as well as Interleukin 1B (IL1B) [18,29]. NFKB1 stimulates the transcription of miR-29b-1 which inhibits extracellular matrix regeneration [18]. These inflammatory molecules attract macrophages and neutrophils which in turn degrade thermally injured tissues. *Tnf* is directly inhibited by Nlrp3 (NLR family pyrin domain containing 3) [38,42] as well as insulin-like growth factor 1 (Igf1) [43]. Hepatocyte growth factor (Hgf) stimulates the expression of Tnf [44], interleukin 1b (Il1b) [44] and apoptosis regulator Bcl-2 (Bcl2) [44]. Bcl-2 is stimulated by Igf1 [45] which activates multiple inflammation reactive molecules e.g. interleukine-4 (Il4) [43], transcription factor AP-1 (Jun) [45] and lymphotoxin-alpha (Lta) [43]. Furthermore, Igf1 inhibits different molecules such as apoptosis regulator Bax (Bax), Nfkb1 [45] and caspase-3 (Casp3) [45,46] in order to hold the baseline between destruction of injured tissue and proliferation as well as regeneration of new matrix.

This review unveiled some unexpected molecular factors which may link the established pathways and key factors.

One unexpected gene product is the profibrotic Protein flightless-1 (FLII) [25]. FLII stimulates the expression of ACTA1 as well as TGFB1 and COL1A1. ACTA1 expression is stimulated by Krueppel-like factor 4 homolog (KLF4) [47] whose expression in turn is stimulated by miR-145 [47]. TGFB1 stimulates the expression of miR-145 [47]. This simple pathway closes the arc between two key factors during burn wound healing. FLII as well as KLF4 are molecular factors which now surprisingly come to the fore. This insight gives new input to international molecular research. Does an inhibition of miR-145 have an antifibrotic effect to burn wound healing while downregulating the expression of KLF4?

FLII as well as SMAD3 stimulate the expression of COL1A1 [20,25]. It is known that SMADs regulate receptors and are activated by TGFB1. More unknown is the connection between SMAD3 and Fibronectin (FN1) [20,29]. FN1 is also stimulated by PDRN. PDRN is able to increase the expression of EPO (Erythropoietin) which is one of the most important inhibitors of the expression of ACTA1 [32,38,48]. This control loop shows the interaction between the key factors COL1A1 and ACTA1. Focused on FN1, PDRN and EPO new hypotheses can be set up. Does the stimulation of EPO help to improve wound healing and reduce scar formation?

Like ACTA1 and COL1A1, TGFB1 is also stimulated by FLII [25]. TGFB1 is able to increase the gene expression of INHBA (Inhibin beta A chain) [34] which leads to increase of AKT1 (RAC-alpha serine/threonine-protein kinase) [34]. AKT1 is a profibrotic kinase which is part of several cell processes and stimulates gene expression of ACTA1 [34]. It forms a link between the key factors TGFB1 and ACTA1. Focusing on the binding element AKT1, new research starting points targeting the support of the wound healing process and the inhibition of scar formation are possible.

FLII is able to stimulate all three key factors and shows negative effects on wound healing and hypertrophic scar formation. Because of its stimulation possibilities, inhibition of FLII could help to reduce wound healing complications and pathological scars. This pathway gives the possibility to increase the molecular importance of single binding elements during burn wound healing. New hypotheses can be generated in order to support the wound healing process while inhibiting pathological scar formation.

5. Conclusion

This systematic review gives a comprehensive overview of molecular interactions and network connections during burn wound healing. It is based on experimentally validated molecular interactions or gene/protein regulations from all included articles. All extracted data about molecular pathways were visualized via PathVisio and published on WikiPathways. Subsequent network analysis via Cytoscape showed new potential molecular interactions across species including humans, mice and rats. However, it has to be pointed out that the presented network is purely based on the gene/protein expression data of the selected literature and thus, the bioinformatics overlay of the individual studies has to be experimentally validated. This network shows a selection of current research and will be published interactively online. Molecular interactions that were previously known and classified as insignificant, are now moving into focus. Single genes like FLII, KLF4, FN1 and AKT1 become more important for further molecular research. Moreover, our manually curated molecular network can be utilized as a hallmark gene set for single-cell-RNA sequencing and Omics data. In summary, this review represents a research snapshot of molecular interactions during burn wound healing. To stay up-to-date, this review is still in progress and can constantly be modulated by researchers worldwide. Systems biology will play an increasingly important role in the future in understanding such complex systems and immense amounts of biological data. Our work facilitates the validation of former hypotheses as well as the generation of new hypotheses regarding the molecular biology of burn wound healing.

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Conflict of interest

The authors have declared that no competing interests exist.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.burns.2023.03.006.

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Attachments to Article III	
 **WikiPathways**	**Homo sapiens:** https://www.wikipathways.org/pathways/WP5055.html
	Mus musculus: https://www.wikipathways.org/pathways/WP5056.html
	Rattus norvegicus: https://www.wikipathways.org/pathways/WP5057.html
	Sus scofa: https://www.wikipathways.org/pathways/WP5058.html
 **Cytoscape**	

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Just saying':

Who says that my dreams have to stay just my dreams?

(Walt Disney. Ariel)

Thank you.