SUMMARIZING DISCUSSION
A scar is an expected result of wound healing. In some individuals, and particularly in burn victims, the wound healing processes may lead to a fibrotic hypertrophic scar, which is raised, red, inflexible and responsible for serious functional and cosmetic problems. It seems that a wide array of sequential processes are involved in hypertrophic scar formation, including an exaggerated inflammation, prolonged reepithelialization, excessive extracellular matrix production, augmented neovascularization, atypical extracellular matrix remodeling, and reduced apoptosis. Platelets, macrophages, T lymphocytes, mast cells, Langerhans cells, and keratinocytes are directly and indirectly involved in the activation of fibroblasts, which in turn produce excess extracellular matrix. Increasing evidence suggests that immunological responses in the early stages following wounding play an important role.

Research into the biological nature of scars has lead to an increased understanding of mechanisms in hypertrophic scar formation (chapter 2), resulting in the development of more specific therapeutic options. Despite this, hypertrophic scars remain difficult to treat. In chapter 3 multiple therapies and studies are discussed. The aim of this thesis research was to identify factors, both at a clinical and molecular level, that are involved in human hypertrophic scar formation. For this we employed several studies investigating patient characteristics and patterns of gene and protein expression of various cytokines and chemokines in scar tissue samples that are associated with hypertrophic scar formation.

Susceptibility to hypertrophic scar formation

Over the past decades, a considerable amount of research has been performed concerning the predisposition and risk factors for keloid formation. Little is known about specific risk factors for hypertrophic scar formation. Furthermore, most studies concerning hypertrophic scar formation are not homogeneous and often include burn patients in an unstandardized fashion. Consequently, estimates of prevalence in these studies vary widely, and knowledge of risk factors for hypertrophic scarring is poor. The identification of patient-related risk factors is particularly valuable to define a high-risk population and may help in planning experimental studies. With this in mind, we described the incidence of hypertrophic scar formation and investigated its association with several patient characteristics in standardized wound healing models (chapter 4).

In our series 60% of patients developed a hypertrophic scar after surgery, typically during the first three months after surgery. Most hypertrophic scars that were present three months after surgery were still hypertrophic after 12 months. Young, non-smoking patients were most susceptible for hypertrophic scar formation. These data contribute to the determination of a population that is at high-risk for developing a hypertrophic scar, and underline the importance of stratifying for age and smoking when analyzing experimental studies on the treatment of hypertrophic scars. Furthermore, in the future these data can contribute to the development of an accurate prediction model, by which clinicians can predict the scar outcome of patients.
**Epidermal versus dermal abnormalities in hypertrophic scars**

Besides an increased incidence in predisposed individuals and susceptible regions of the body, hypertrophic scar formation occurs more often after delayed reepithelialization due to deep wounding or wound infection. Although it is mainly described as a disorder of the dermis, histologic evaluation of hypertrophic scar tissue has also revealed epidermal abnormalities. Increased numbers of activated keratinocytes are present in young and mature hypertrophic scar tissue but not in normal scars. Activated keratinocytes express keratin 6 and keratin 16 and are normally found mainly during the proliferative phase of wound healing. They show augmented biochemical activity, such as an increased proliferation rate and increased production of cytokines, thereby intensifying their communication with other cells. Also, hypertrophic scar formation is associated with an increased number of epidermal Langerhans cells, which have an antigen-presenting function.

Keratinocyte and Langerhans cell abnormalities in hypertrophic scars strongly resemble epidermal alterations observed in the skin disorder psoriasis. Psoriasis can be effectively treated with topical application of calcipotriol, a synthetic derivative of vitamin D. Calcipotriol stimulates keratinocyte terminal differentiation, inhibits keratinocyte proliferation, and suppresses the number and antigen-presenting function of epidermal Langerhans cells. Moreover, it has anti-inflammatory potency. Considering all of this, we hypothesized that topical application of calcipotriol could prevent hypertrophic scar formation by altering the biochemical properties of the epidermis. Therefore, a randomized, double-blind, placebo-controlled trial was performed to investigate the preventive effect of topical application of calcipotriol on hypertrophic scar formation. Furthermore, the biochemical properties of the epidermis during hypertrophic scar formation were investigated (chapter 5).

We established that topical application of calcipotriol during the first 3 months of wound healing does not affect the incidence of hypertrophic scar formation. Furthermore, we observed a strong association between keratinocyte activation and hypertrophic scar formation. This prolonged activated epidermis is associated with reduced interleukin 1α and increased profibrotic platelet-derived growth factor expression compared with normal scar tissue, which may directly affect fibroblast collagen production. What causes the keratinocytes to remain activated is not completely clear, but because the activated phenotype is generally present only during the inflammatory phase of wound healing, and because increased persistence of epidermal Langerhans cells has been observed in hypertrophic scars, it seems that a prolonged inflammatory phase is an important prerequisite for, or sign of, hypertrophic scar formation. During this prolonged inflammatory phase, pro-inflammatory cytokines derived from macrophages most likely keep the keratinocytes activated, whereas fibroblasts remain stimulated to produce extracellular matrix.

**Inflammation and hypertrophic scar formation**

Currently it is not known at what moment during hypertrophic scar formation the normal wound healing process derails. Nonetheless, evidence is increasing that
immunologic responses manifested shortly after wounding play an important role in hypertrophic scar formation\textsuperscript{3,4}.

Corticosteroids are widely used as treatment for excessive scarring by intrallesional injection\textsuperscript{20,21}. They have immunomodulating and anti-inflammatory effects by reducing pro-inflammatory cytokines, adhesion molecules, and inflammatory enzymes\textsuperscript{22}. Multiple studies have proven the efficacy of corticosteroids for treatment of existing hypertrophic scars\textsuperscript{23-25}. Considering this, the use of corticosteroids to prevent hypertrophic scar formation seems rational. However, recurrence rates of 9\% to 50\% are observed when corticosteroids are injected intrallesionally subsequent to scar resection\textsuperscript{20}. It is conceivable that systemically administered corticosteroids affect a wider range of inflammatory processes that influence wound healing and may therefore be more successful in preventing hypertrophic scar formation.

To study this assumption we conducted a prospective cohort study in patients undergoing cardiothoracic surgery through a median sternotomy incision (chapter 6). The presternal scars caused by this operation have a high incidence of hypertrophic scar formation\textsuperscript{26}. Dexamethasone was administered systemically at high dose before and after cardiac surgery that required the use of cardiopulmonary bypass. To evaluate the effect of the dexamethasone, both patients who did and who did not receive dexamethasone perioperatively were examined 2, 4, 6, 12, and 52 weeks postoperatively. The presternal scars were clinically evaluated, measured and scored as normotrophic or hypertrophic.

We observed no statistically significant differences in incidence of scar hypertrophy when comparing patients who received and who did not receive dexamethasone perioperatively. However, scars became significantly wider in the dexamethasone group compared with the control group. Furthermore, twelve weeks postoperatively scars were significantly thicker in the dexamethasone group. These differences in scar width and thickness between the dexamethasone and control group were mainly present in patients that developed hypertrophic scars. These results suggest that administration of high-dose perioperative dexamethasone does not prevent hypertrophic scar formation. Its use together with the cardiopulmonary bypass, however, did affect scar dimensions negatively up to 52 weeks after surgery. An explanation for these findings could lie in the effect of the cardiopulmonary bypass, which activates the inflammatory response, or in the effect that corticosteroids have on macrophage activation and differentiation. Corticosteroids are known to stimulate the alternative activation of macrophages, which differentiates them into a profibrotic phenotype\textsuperscript{27}.

Macrophages are considered key players in wound healing, as they serve as a source of various cytokines and chemokines essential for orchestrating the wound healing process and ECM production. Since the role of macrophage phenotype has been highlighted in fibrosis in several studies, while other studies underline the importance of macrophages in wound healing, the interest in macrophage function in wound repair is rapidly increasing.

Tissue macrophages arise from monocytes and can display different functional phenotypes. Mediators that stimulate macrophages to differentiate into different functional phenotypes are typically derived from activated T helper cells\textsuperscript{28}. The currently most widely used classification scheme for the activation of macrophages defines
classically activated macrophages as M1, and the group of non-classically activated (or alternatively activated) as M2. M1 macrophages have an increased expression of CD40 and exhibit antimicrobial properties releasing inflammatory mediators including tumor necrosis factor (TNF)-α, nitric oxide (NO) and interleukin (IL)-6 and IL-12. M2 macrophages produce TGF-β and IL-10 and express both mannose receptor (CD206) and haemoglobin scavenger receptor (CD163). They are involved in the defense against parasitic infections, but also contribute to allergy, asthma and fibrosis. And more specifically, a relatively high expression of cytokines such as TGF-β1 and IL-4, associated with an M2 phenotype, is associated with hypertrophic scar formation.

The above-mentioned macrophage phenotypes are actually extremes of a continuum of macrophage function and have not been determined in pure form in vivo. The current literature lacks in vivo characterization of the receptor expression and cytokine production kinetics by macrophages during cutaneous wound healing in humans. Thus, whether the proposed phenotypes actually represent macrophage populations in the human host, and how these phenotypes are represented in the human wound during normotrophic and hypertrophic scar formation, is currently unknown. Therefore, we investigated the cellular influx of inflammatory cells and especially the macrophage phenotype associated with normotrophic and hypertrophic scar formation in time (chapter 7).

Human presternal wound healing after cardiothoracic surgery through a sternotomy incision was investigated in a standardized manner. Skin/scar biopsies were collected at six time points; during surgery (control sample), 2, 4, 6, 12, and 52 weeks postoperatively. The number and distribution of CD45+, CD68+, CD40+, CD163+ and CD206+ cells were evaluated. Expression levels of the genes associated with classically- and alternatively-activated macrophages were investigated.

Our data shows that in scar tissue samples developing to hypertrophic scars an increased number of macrophages is present 4 and 6 weeks postoperatively. These macrophages have an increased expression of CD163 and CD40, which are known to be markers for M1 and M2 macrophages, respectively. The expression of mannose receptor (CD206), another M2 marker, was expressed similarly in both groups, with a slightly higher expression 2, 4 and 6 weeks postoperatively compared with the control samples. The expression levels of typical M1- or M2-associated cytokines were not significantly higher in the hypertrophic group at these specific time points where we observed a higher number of CD163+ and CD40+ macrophages. Therefore, an association between hypertrophic scar formation and a macrophage phenotype that fits the current dogmas on macrophage activation and differentiation could not be established in these human tissue samples. It should however be noted that TGF-β1 mRNA was expressed at higher level in the hypertrophic group 2 weeks postoperatively, which could be an effect attributed to the early inflammatory phase.

As our approach was descriptive and the role of alternatively-activated macrophages in hypertrophic scar formation is still speculative, further studies will need to be performed to analyze the contribution of alternatively-activated macrophages. This will hopefully provide us with a better understanding of the actual macrophage phenotype and function during normal human wound healing and wound healing.
associated with hypertrophic scar formation. Before intervention in these complex processes is justified, more knowledge of the role of macrophages in human cutaneous wound healing is necessary, in order to establish their functional phenotype in vivo in the different phases of wound repair and (hypertrophic) scar formation.

Angiogenic response during hypertrophic scar formation

New blood vessel formation is an essential process in wound healing that is thought to mainly manifest as angiogenic sprouting of pre-existing capillaries. The new vessels invade and re-populate the wound in unison with macrophages, keratinocytes, and fibroblasts as a source of new extracellular matrix constituents and a plethora of cytokines and growth factors. Compared with normal skin, hypertrophic scars have an increased number of vessels with a more dilated phenotype. Furthermore, in hypertrophic scars the blood flow is increased as measured by laser-doppler. This implies the presence of an increased vascular density in hypertrophic scars compared with normotrophic scars.

Angiogenic sprouting involves different mediators that are spatiotemporally expressed in a highly controlled manner. The Angiopoietins comprise a family of angiogenic mediators that regulate vascular stability, neovascularization, and vascular maturation. Angiopoietin (Ang)-1 is constitutively expressed by vascular support cells and binds to Tie-2, a transmembrane tyrosine kinase receptor present on endothelial cells. Ang-1 mediated Tie-2 signaling maintains endothelial cells in a quiescent state, thereby limiting endothelial activation by factors like vascular endothelial growth factor (VEGF). Vascular destabilization is created by inflammatory stress induced endothelial release of Ang-2 that competes with Ang-1 for Tie-2. As a consequence, the endothelium becomes prone to VEGF-induced VEGF-receptor (VEGFR) signaling. VEGF is considered to be instrumental for endothelial cells to further engage in new sprout formation, as it has a direct effect on vascular permeability and temporary fibrin matrix formation, microvascular endothelial cell migration, proliferation, and survival. In order to migrate and produce new capillaries, endothelial cells furthermore upregulate a variety of proteases including the fibrinolytic protease urokinase-type plasminogen activator (uPA) by means of which they can degrade the basement membrane and extracellular matrix. How these processes are regulated during wound healing associated with hypertrophic scar formation is currently unknown.

To provide further insight in, and to identify new molecular details about the angiogenic profile during normotrophic and hypertrophic scar formation, we examined the time course of the angiogenic response during hypertrophic scar formation in humans in comparison with normotrophic scar formation (chapter 8).

In our study microvessel densities were significantly higher in the hypertrophic compared with the normotrophic scars 12 weeks and 52 weeks postoperatively. Ang-1 expression was lower in the hypertrophic group, which, together with a nonsignificant increase of Ang-2 expression, represented a considerable decrease in the Ang-1/Ang-2 ratio in the hypertrophic group 4 weeks, 12 weeks, and 52 weeks postoperatively. Expression of uPA was upregulated during hypertrophic scar formation. VEGF expression
was not significantly different when comparing both groups, but we observed a highly variable expression in the hypertrophic group after 6 weeks.

In summary, we established that differential expression of Ang-1, Ang-2 and uPA in time is associated with an increased vascular density in hypertrophic scars compared with normotrophic scars. Future studies will address whether the increased neovascularization during hypertrophic scar formation is a cause or a consequence of the pathological condition, and whether the differential expression of angiogenic factors may be a suitable target for (preventive) treatment of hypertrophic scar formation.

Concluding remarks and future directions

Research on the molecular and cellular mechanisms of wound healing and scar formation has lead to a better understanding of mechanisms that are possibly involved in hypertrophic scar formation. It seems that a wide array of wound healing processes is involved in hypertrophic scar formation, and once the hypertrophic scar trail is taken, the involved cells jointly create and maintain an environment that promotes the scar phenotype. We identified that smoking and age influence the incidence of hypertrophic scar formation. Further research should address the actual biochemical effect of smoking on scar formation, and the identification of other patient characteristics, genetic factors and immunological responses that influence hypertrophic scar formation, which can be used to predict its incidence and regression. From our studies we can furthermore conclude that immunologic responses that occur very shortly after wounding are involved in the determination of the trail that will be taken for months. Our findings significantly altered the way we think about the timing of the administration of potential therapeutic agents that intervene in the wound healing and scar formation process.

Despite the new insights in molecular and cellular abnormalities associated with hypertrophic scar formation, a high-quality therapy to prevent hypertrophic scar formation is still lacking. This may be a consequence of poor extrapolation of observations in in vitro cell systems and animal models to the wound in a patient. In studies investigating wound healing, and especially the inflammatory response, interactions both through cell-cell contact and through secreted products, are spatiotemporally controlled and difficult to mimic in in vitro cell systems. Furthermore, wound healing in other species presents significant differences when compared with wound healing in humans, and hypertrophic scar formation is a condition that naturally only occurs in humans. Hence, studying cell (dys)function in the complex microenvironment of the human wound remains a prerequisite. The knowledge created in simplified in vitro, ex vivo, or animal models in conjunction with observations in patients can help to more closely define which molecular and cellular features are implicated in aberrant wound healing in humans.

It remains unclear whether the currently identified molecular and cellular abnormalities associated with hypertrophic scar formation are a cause or a consequence of the unusual scar tissue formation. Research on this topic should change its focus to the early inflammatory response during the wound healing process before the hypertrophic scar has developed. Even before the actual wounding has taken place,
people may have predisposed differences in cytokine expression which promotes hypertrophic scar formation. In this thesis we have identified several leads that the phenotype of the immunological response early following wounding is important for the eventual scar outcome. Local immune response influence both the quantity and phenotype of inflammatory cells and mediators that migrate into the wound area, which are responsible for the orchestration of the wound healing and scar formation process. In my opinion, macrophages play the key role here.

Alternatively-activated macrophages have been linked to fibrosis, where they are thought to play a major role in the early stages of disease by producing profibrotic cytokines. Although it is very likely that different macrophage phenotypes influence ECM production differently, the actual presence of the extremes of the possible differentiation routes of macrophages that have been identified in vitro has not been established in vivo. Although we established that scars developing towards hypertrophic scars display an increased number of macrophages 4 and 6 weeks after injury, we did not find an association between hypertrophic scar formation and a macrophage phenotype that fits the current theories on macrophage activation and differentiation. Our findings are limited for several reasons. For example, we were not able to collect tissue samples between surgery and 2 weeks after surgery, during which most inflammatory processes take place, and which is believed to be the phase were the stage is set for the wound healing and scar formation process. Furthermore, we analyzed mRNA expression of cytokines using total RNA from the specimen biopsies. Possibly this method of analyzing gene expression is not sensitive enough to pick up differences in gene expression of inflammatory cytokines. In the future, laser capture microdissection followed by genomic and proteomic analysis of macrophages from human wound biopsies will be helpful in determining the expression of cytokines produced by macrophages more specifically, and subsequently to determine the macrophage phenotype in vivo.

Besides inflammation, angiogenesis-related processes also seem to be involved in hypertrophic scar formation. The finding that hypertrophic scars have an increased number of blood vessels, and that the expression levels of Ang-1, Ang-2 and uPA were changed during hypertrophic scar formation compared with their levels during normotrophic scar formation support this hypothesis. However, many issues remain to be solved regarding the relation between angiogenesis and vascular remodeling, and hypertrophic scar formation. The first is whether increased neovascularization accompanying hypertrophic scar formation is a cause or a consequence of the pathological condition. Various studies have addressed the role of angiogenesis in wound healing processes per se in animal models, yet data on its relation to hypertrophic scar formation are scarce. The second issue deals with the intriguing possibility that a unique molecular pathway of neovascularization is responsible for the excessive vascularization in hypertrophic scar tissue formation. The observation that inhibition of VEGF-mediated VEGFR signaling via various methods does not affect wound healing in general implies that redundant pathways may be able to take over the control of new blood vessel formation during the wound healing process. Furthermore, it was recently discovered that biomechanical forces can induce vessel translocation by a largely VEGF-independent mechanism of vascularization.
during wound healing\textsuperscript{53}. Possibly, these pathways are also adaptive to the dynamics of changing conditions during wound healing and scar tissue formation and as such directly involved in excessive new vessel formation and scar hypertrophy induction, and this should be subject to further investigation. The third issue relates to the differences in vascular behavior throughout the vascular tree. Possibly in wound healing the molecular fine-tuning of angiogenesis varies depending on the site where the wound is located, which correlates with the observation that hypertrophic scar formation is more common in certain parts of the body.

Many therapeutic options have been developed to treat existing hypertrophic scars. To compare these treatments individually or a combination of treatments properly, which is crucial in order to establish the best methods for managing hypertrophic scars in daily practice, it is of the utmost importance that consensus concerning the definition of a hypertrophic scar, according to an assessment tool, will be developed and internationally used. My recommendation regarding hypertrophic scarring in the daily practice is to classify and assess the scar by means of the POSAS, available at www.posas.org. Scars with a POSAS thickness score of $\geq 3$ can be defined as hypertrophic. Regarding the assessment of scars in clinical trials, I recommend the use of the POSAS and additional documentation of the percentage of the original wound surface area that is involved. When investigating the incidence of hypertrophic scarring or when assessing treatment effectiveness, the following data should be taken into account, in order to further identify risk factors for hypertrophic scar formation: skin type, age, location of the wound, wound depth, time of healing, treatment, time of surgery, wound area and hypertrophic scar area (percentage of the original wound area with hypertrophy according to POSAS). In this way, a reliable comparison of new and existing therapies can be made, in order to establish the best treatment possible for those who need it.

The ultimate goal of the research performed in this area is, of course, to develop treatment modalities to prevent hypertrophic scar formation. On molecular level, these therapeutics will need to intervene in signal transduction cascades in order to influence the process that leads to the formation of a (hypertrophic) scar. More studies investigating cell (dys)function and cytokine expression in human wounds and scars will be needed to exactly define which molecular and cellular features are implicated in hypertrophic scar formation in humans. Furthermore, a suitable animal model will need to be developed, so that the information that comes from human studies can be translated to an \textit{in vivo} model, and experimental treatments can be tested more easily.

In the future, this successful preventive treatment will be administered to susceptible patients, identified by genetic factors and immunological markers, quickly after wounding and will be able to control the phenotype of the inflammatory response, particularly the phenotype of the macrophages in the wound bed, in order to influence the subsequent continuous and overlapping wound healing processes and put them on track towards the formation of a normal scar. But we are not there yet. Further research on patient characteristics, genetic factors and immunological responses that influence hypertrophic scar formation, and cellular and molecular mechanisms of wound healing and hypertrophic scar formation is crucial in order to be able to influence the evolution of hypertrophic scars towards normal skin in the future.
REFERENCES
