Hamstring tendon regeneration following harvest for anterior cruciate ligament reconstruction

Mathijs A.M. Suijkerbuijk

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CHAPTER 1

General introduction,
thesis aim and outline

ANTERIOR CRUCIATE LIGAMENT RUPTURE AND TREATMENT

ACL rupture is a common sports-related injury potentially causing instability of the knee joint. In the general population, annual incidence rates reach up to 5 - 8 per 10.000 persons[1, 2]. On the contrary, incidence rates reported for professional athletes are substantial higher: 8 to 52 per 10.000 per persons per year in various populations including Sweden, Norway, Denmark, The United States of America, Australia and Germany[1]. However, the exact incidence in The Netherlands is unknown. ACL injuries are most frequently observed in pivoting sports, such as down-hill skiing, soccer, handball and basketball[3]. Women are at 2 to 8 times greater risk as men of suffering this injury[4, 5].
Currently, the treatment options are either a conservative regime with exercise therapy or a surgical reconstruction of the injured ACL. The Dutch ACL guidelines recommend surgical reconstruction only when knee instability exists. Otherwise, a conservative treatment is indicated[6]. When despite adequate conservative therapy complaints of instability remain, one might consider operative treatment too. Other factors that contribute to the final treatment decision are additional injuries and patient's requirements in terms of activity levels and participation in pivoting sports[6, 7]. The number of ACL reconstruction procedures performed globally and in The Netherlands is increasing[8, 9]. The estimated number of ACL reconstructions in The Netherlands in 2003 was 3.000[9], whereas today's estimations reach up to 7.000 reconstructions annually[10].
An important aspect of the ACL reconstruction procedure is the graft choice. Today, several graft options exist, including autografts, allografts and synthetic grafts. Because of unlimited access and no donor-site morbidity, synthetic grafts were popular in the past. However, these grafts presented serious drawbacks such as immunological responses, recurrent instability and knee osteoarthritis[11]. Therefore, artificial grafts are hardly used in current clinical practice[12]. There are various allografts available for reconstruction purposes, such as tibialis posterior tendon, tibialis anterior tendon, Achilles tendon, peroneus longus tendon and bone-patellar tendon-bone (BPTB). A potential disadvantage of the use of allografts is the risk of infection, graft rejection and graft elongation. These disadvantages are less likely to occur in autografts. Autografts are therefore the most preferred graft for ACL reconstruction procedures. The most commonly used autografts are the hamstring tendons and bone-patellar tendon-bone (BPTB)[13]. As BPTB grafts are associated with donor-site morbidity in 80% of the patients[14] and patellar tendon rupture occurs in 0.24%[15], the hamstring tendon autografts are the graft of choice to replace injured ligaments in the Netherlands as well as globally. Orthopaedic surgeons tend to harvest two hamstring tendons and subsequently fold them to create the typical 4-stranded graft. This ensures that an optimal graft size is obtained and so that optimal biomechanical function is reached[16].

LIGAMENTS AND TENDONS

Anatomy

The anterior cruciate ligament (ACL) is a ligament that courses from the femur to the tibia. More precisely, the ACL arises from the posteromedial side of the lateral femur condyl and attaches on the anteromedial side of the tibia plateau (Figure 1). The ACL is comprised of two bundles named for their insertion sites on the tibia plateau: anteromedial (AM) bundle and posterolateral (PL) bundle[17]. Its main function is considered the primary restraint to anterior displacement of the tibia and to provide rotational stability[18].

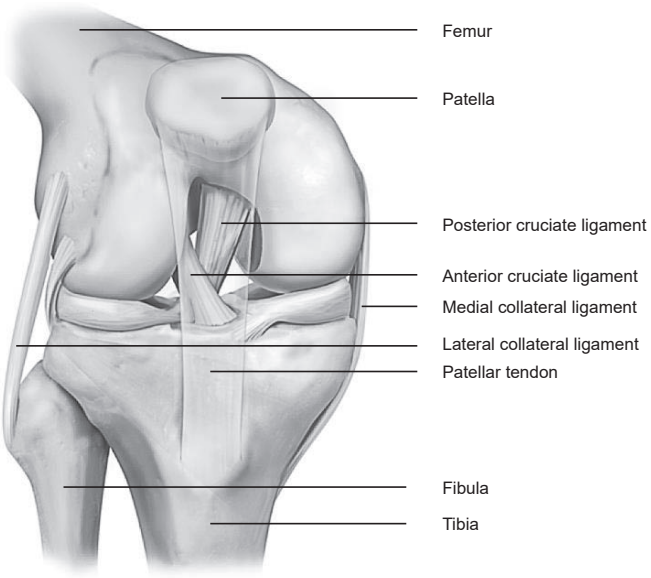

Figure 1: Anatomical representation of the right knee (modified from Kennedy et al.[19]).

The two tendons that are harvested for reconstructive purposes are the semitendinosus and gracilis tendons. The semitendinosus is located in the postero-medial side of the thigh and has its origo at the inferior-medial aspect of the ischial tuberosity. The proximal tendon shares a tendon with the biceps femoris. The long distal tendon, which is harvested for reconstruction of the ACL, starts caudal from the mid-thigh.

The gracilis tendon has it origo at the ramus inferior ossis pubis and descends along the medial thigh. From an anatomical and functional perspective, the m. gracilis is considered to be an adductor of the leg. The tendons of the semitendinosus, gracilis and sartorius eventually conjoin to form the pes anserinus. The pes then turns around the medial aspect of the tibia and inserts at the tuberositas tibiae.

It should be noted that in the light of autografts for ACL reconstructions, the m. semitendinosus and m. gracilis are often referred to as hamstring tendons.

Structure

Tendons and ligaments are hierarchically organised. The main structural component is collagen, which is a triple helix. The assembly of five collagen molecules is termed a microfibril. These microfibrils are arranged into larger longitudinal bundles. Depending on their size, these bundles are called subfibrils, fibrils and fascicles (Figure 2). Each fascicle is separated by a layer of loose connective tissue that is known as the endotenon. A group of fascicles form the entire tendon, which is enclosed by the epitenon: a connective tissue-sheath containing the vascular, lymphatic and nerve supply. The ligamentous equivalent for endotenon is endoligament, whereas epitenon is referred to as epiligament. In general, the collagen fibers are organised in the direction of the applied force. As forces in tendons are applied in a uniaxial direction, a parallel alignment of the collagen fibrils is found in tendons. However, collagen fibrils are not as uniformly orientated in ligaments because forces are applied in more than one direction.

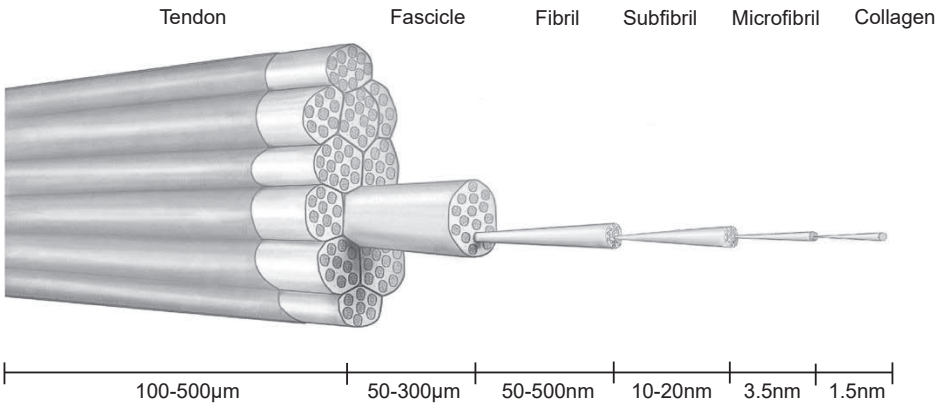

Figure 2: Schematic image of the hierarchical structure of tendons and ligaments (modified from Encyclopedia Britannica[20]).

Composition

The extracellular matrix (ECM) of tendons and ligaments is approximately composed of 65-80% collagen (dry weight)[21-23]. Collagen type I is with 95% of the total collagen the predominant collagen in both ligaments and tendons. Additionally, at least 28 more collagen types are found in minimal concentrations[24, 25]. Collagens contribute to the structural framework in tendons and ligaments as they form both intramolecular and intermolecular covalent cross-links. This stabilises the ECM and determines its tensile strength. Forms of cross-linking that are generally found in tendons and ligaments are the hydroxylysine aldehyde derived and the lysine aldehyde derived cross-links[26]. These are established after enzymatic modifications[27]. Another mechanism of cross-linking is via non-enzymatic modifications using glucose, with pentosidine as a well-identified end product[28].

Although collagen fibrils are the main component in the ECM of tendons and ligaments, several other non-collagenous constituents also contribute to its overall function. Proteoglycans, a special class of glycoproteins, represent 3% of the dry weight in tendons and ligaments[29, 30]. These proteoglycans contain glycosaminoglycan (GAG) subunits that, due to their high concentration of negative charge, generate an osmotic pressure by attracting water. The water content of the matrix is about 70% of the wet weight of the ECM. This leads to lubrication and spacing allowing fibers to glide over each[31].
Highly specialized fibroblasts are sparsely present in the ECM, but represent the main cell type in tendons and ligaments comprising 90-95% of the cell population[32, 33]. These fibroblasts in tendons are referred to as tenocytes and in ligaments as ligamentocytes. These cells are involved in the degradation and synthesis of ECM components.

TISSUE HEALING AND INFLAMMATION

It has been reported that hamstring tendons harvested for ACL reconstruction are able to regenerate after surgical resection[34]. These regenerated tendons clinically appear as a well-defined fibrous band that could be palpated on the posteromedial aspect of the popliteal fossa[35]. Macroscopically, regenerated tendons have the same colour and glossiness as those of normal hamstring tendons[35, 36]. In addition, several studies microscopically examined the regenerated tissue. No significant differences were found in terms of collagen type, fiber structure, cellularity, vascularity and amount of GAGs when comparing the regenerated tendon with native tendon[35-38]. This illustrates the remarkable extent of tendon healing following harvesting procedures.
Tissue healing is a complex and multistage process, involving the recruitment of various cells. These cells typically produce their own cytokines or growth factors contributing to the process of tissue healing. Tissue healing can be subdivided in four stages[39]:

1. Haemostasis: the blood clotting system is activated in the first minutes to hours after (iatrogenic) injury. More specifically, thrombocytes and platelets aggregate in a fibrin network[40]. Additionally, these platelets release cytokines and growth factors to attract other cells.
2. Inflammation: during this phase inflammatory cells are recruited to remove dead cells, bacteria and other pathogens. Together with macrophages, mast cells and T-lymphocytes are attracted and subsequently secrete multiple factors to influence the process of tissue healing[41]. This process typically takes a few days to a few weeks.
3. Proliferation: following the inflammatory phase, cells will start to proliferate and synthesize structural and fibril-associated components of the extracellular matrix. This step can take a few days up to weeks after injury.

4. Remodelling: in this final stage, the new tissue will be rearranged into normal tissue structure. In particular the orientation of collagen fibers is reorganised along the tension lines. Furthermore, superfluous cells will undergo apoptosis during this phase. In general, this phase might take weeks to months following tissue injury.

Macrophages are a major component of the mononuclear phagocyte system and are key role players in the inflammatory phase of the process of tissue healing[42]. These specialized cells of the immune system are derived from monocytes. Depending on the microenvironmental cues, macrophages are able to obtain a whole spectrum of different phenotypes with distinct functional and phenotypical characteristics[43, 44]. The tissue-remodelling process is orchestrated by these macrophages, but more specifically by their produced cytokines and chemokines[41].

Pro-inflammatory macrophages, or M1, represent one end of the spectrum. Their main function is to debride affected sites by phagocytosis of pathogens, foreign materials and damaged cells[45]. Also, pro-inflammatory macrophages are responsible for the production of numerous pro-inflammatory cytokines including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α. Conversely, on the other end of the spectrum, anti-inflammatory macrophages, or M2, are found. They are involved in tissue repair and healing processes by the secretion of anti-inflammatory cytokines, such as IL-4, IL-10 and transforming growth factor (TGF)-β[43, 44]. These cytokines, interleukines and growth factors are known to activate different pathways;

Apoptosis Inflammatory factors are known to stimulate the production of reactive oxygen species, resulting in the production of caspases[46, 47]. Caspases are known to induce apoptotic cell death[23]. A decrease in cell numbers directly compromises maintenance and repair of the ECM, as cells are responsible for the production of ECM components.

Fibrosis Other inflammatory factors contribute to an upregulation of TGF-β leading to an increased production of collagens and proteoglycans[48-51]. Ultimately, this might lead to fibrosis. In addition, TGF- β induces the synthesis of tissue inhibitors of metalloproteinases (TIMPs), preventing the degradation of matrix components[52].

ECM degradation The production of prostaglandin E_2 is induced following exposure to inflammatory cytokines and leads to an upregulated production of metallaproteinases (MMPs)[53-55]. These proteins are known to enhance the degradation of ECM components. Taken together, the inflammatory response is a complex combination of pro- and anti-inflammatory factors that needs to be tightly regulated. An imbalance between the pro- and anti-inflammatory response leaves the inflammation unchecked resulting in either too much matrix degradation or too much fibrotic tissue.

AIMS AND OUTLINE OF THIS THESIS

Anterior cruciate ligament (ACL) reconstruction has become standard orthopaedic practice worldwide and often requires harvest of the hamstring tendons. However, the harvest of functional and healthy tissue might lead to donor-site morbidity and functional deficits. In 1992, Cross et al. were the first ones to describe the remarkable feature of these tendons to regenerate following harvesting procedures, potentially solving the post-harvest morbidity[34]. Therefore, the general aim of this thesis is to improve the outcome after hamstring tendon harvesting through a better understanding of tendon regeneration.

In **Chapter 2** we conduct a systematic review to summarize the available literature about hamstring tendon regeneration following harvesting procedures.

Regeneration of the hamstring tendons has been associated with various clinical symptoms, such as pain in the posterior thigh, cramping and muscle weakness[56]. These symptoms might be explained by failure of the regeneration process or altered morphological properties of the regenerated tendons. **Chapter 3** describes the process of hamstring tendon regeneration at one- and two-years follow-up after ACL reconstruction entailing the hamstring tendons using magnetic resonance imaging. More specifically, it reports regeneration rates, changes in cross-sectional areas and tendon lengths.

Considering the clinical symptoms, it might be interesting to preoperatively identify patients that are likely to lack a regenerative capacity of the hamstring tendons. Knowledge about modulators for hamstring tendon regeneration might alter the graft choice. Therefore, **chapter 4** identifies predictive factors for hamstring tendon regeneration. In addition, patient-reported outcome measurements between patients with and without hamstring tendon regeneration are reported.

Inflammation is a well-known factor that contributes to tissue repair. However, a better understanding of the effects of inflammatory factors on the production of extracellular matrix components is required to direct the inflammatory process and to improve tendon regeneration. Currently, it remains unclear how polymorphisms within genes encoding inflammatory proteins such as *interleukin (IL)1B* and *IL6* affect the production of structural and fibril-associated components of the extracellular matrix. **Chapter 5** focuses on the effect of polymorphisms within genes encoding for two inflammatory factors (*IL1B* and *IL6)* on gene expression levels of collagens and proteoglycans in fibroblasts with an increased or decreased injury risk.

Immune cells, in particular macrophages, are the key role players in inflammation and are known to produce inflammatory factors such as IL-1β and IL-6. The production of these proteins is known to be stimulated following activation of a specific signaling pathway. **Chapter 6** describes the effects of specific inhibition of this inflammatory signaling pathway on macrophage phenotypes.

Finally, **chapter 7** summarizes and discusses the main findings and limitations of the studies described in this thesis. In addition, it combines the knowledge of the studies to discuss potential directions for future research in order to improve the outcome following hamstring tendon harvesting procedures.

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CHAPTER 2

Hamstring tendon regeneration after harvesting: a systematic review

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ABSTRACT

Background: Hamstring tendons are often used as autografts for anterior cruciate ligament (ACL) reconstruction. However, no systematic review has been performed describing consequences, such as hamstring tendon regeneration rate and determinants of hamstring tendon regeneration.

Purpose: To summarize the current literature regarding hamstring tendon regeneration rate, the time course of regeneration, and determinants of hamstring regeneration.

Study design: Systematic review.

Methods: A search was performed in the Embase, Medline (OvidSP), Web-of-Science, Cochrane, PubMed and Google Scholar databases up to June 2014 to identify relevant articles. A study was eligible if it met the following inclusion criteria: tendons were harvested, regeneration at harvest site was assessed, population size was at least 10 human subjects, full-text article was available and the study design was either a randomized controlled trial, prospective cohort study, retrospective cohort study or case control study. A risk of bias assessment of the eligible articles was determined. Data describing hamstring tendon regeneration rates were pooled per time period.

Results: A total of 18 publications met the inclusion criteria. The mean regeneration rate for the semitendinosus and gracilis was, in all cases, 70%, or higher. More than 1 year after harvesting, 79% (median [IQR], 80 [75.5-90]) of the semitendinosus tendons and 72% (median [IQR], 80 [61-88.5]) of the gracilis tendons were regenerated. No significant differences in regeneration rate could be found considering patient sex, age, height, weight or duration of immobilization. Results did not clearly show whether absence of regeneration disadvantages the subsequent hamstring function. Five studies measured the regeneration rate at different moments in time.

Conclusion: Hamstring tendons regenerated in the majority of patients after ACL reconstruction. The majority of the hamstring tendon regeneration was found to occur between 1 month and 1 year after harvest. No significant determinants for hamstring tendon regeneration could be identified because of a lack of research. The function and strength of the regenerated hamstring remained unclear.

Clinical relevance: Insight into hamstring tendon regeneration is of clinical relevance as it may influence the choice of ACL graft and it may alter the current rehabilitation after harvesting the tendon.

Key terms: hamstring tendon regeneration; determinants; time course; clinical outcome.

INTRODUCTION

The hamstring has become one of the most often harvested tendons used to reconstruct the anterior cruciate ligament (ACL) after rupture[18]. Hamstring tendon autografts are used more often for primary ACL reconstruction compared with bone-patellar tendon-bone (BPTB) autografts[1,12,17]. This may be the result of several advantages to using hamstring tendons, such as less donor-site morbidity, fewer kneeling problems, and fewer patellar tendon ruptures[8,9,32,33].

In 1992, Cross et al[5] were the first to describe the potential of hamstring tendons to regenerate after harvesting for ACL reconstruction. However, in the following years, after observing neotendons by histology or visual means, investigators found that some hamstring tendons seemed to lack the ability to regenerate[16,27].

Several predictive factors have been identified for tendon regeneration in general. Some examples of determinants that may negatively influence tendon regeneration are the use of nonsteroidal anti-inflammatory drugs[25], the use of nicotine[22], and diabetes mellitus[10,11]. However, no systematic review has described determinants for hamstring tendon regeneration before.

Knowledge of regeneration of hamstring tendons is of clinical importance, as it may influence the choice of ACL graft and may even change rehabilitation programs after surgery[13]. In addition, some patients voice concerns about the consequences of removing native tendons and the functional deficits that may result as a consequence. This systematic review aimed to answer these questions.

No systematic review has been performed concerning the regeneration of harvested hamstring tendons previously, nor has a review been performed to describe determinants for hamstring tendon regeneration. The aim of this systematic review was to summarize (1) hamstring regeneration rate after harvesting, (2) the time course of regeneration, (3) the morbidity and function loss of nonregenerated harvested hamstrings, and (4) determinants that may influence the process of regeneration.

METHODS

Search strategy

The search strategy (Supplementary Table 1) was carried out on published literature from the following electronic databases: Embase, Medline (OvidSP), Web-of-Science, Cochrane, PubMed and Google Scholar. These databases were searched from their inception to June 1, 2014. Additionally, the reference list of each included study was reviewed.

Eligibility criteria

A publication was eligible if (1) a surgical procedure that entailed hamstring tendon harvesting was used, (2) an evaluation of hamstring regeneration at harvest site was performed, (3) the study population consisted of a minimum of 10 patients, (4) the study was performed on humans, (5) full-text article was available, and (6) the study design was a randomized controlled trial, prospective cohort study, retrospective cohort study, or case control study.

Studies were excluded when (1) the outcome was other than specified in the inclusion criteria (e.g. evaluation of the hamstring tendon autograft), (2) there was no information about the regeneration, or (3) previous hamstring injuries were reported.

Animal studies were also excluded. The search was limited for language (English, Dutch, French, German, or Spanish).

Identification of eligible studies

Identified studies were screened, based on title and/or abstract, independently by 2 reviewers (M.S., D.M.). Full-text versions of the selected studies were reviewed, and if they met the eligibility criteria, the study was included in the current systematic review. Disagreements were solved by consensus.

Data extraction

Three independent reviewers (M.S., S.L., and J.P.) performed data extraction from each included publication. Extracted characteristics of the included studies were as follows: number of included subjects, sex, average age, time between surgery and evaluation, imaging technique and experience of examiner. The outcome measures were percentages of tendon regeneration, the time course of regeneration, the morbidity of harvested hamstrings not regenerated, and determinants predicting the regeneration potential of the hamstring tendon. Hamstring tendon regeneration rates are displayed in percentages based on their follow-up periods (less than or more than 1 year).

Risk-of-bias assessment

We assessed the risk of bias of studies using a quality assessment list (Table 1), based on modified questions of existing quality assessment tools[6, 7, 29]. The purposes of this systematic review were of a different nature. Studies reporting the rate of tendon regeneration were considered to have a low risk of bias if consecutive patients were included and if the imaging technique used was valid and reliable. Next to these criteria, in order to be considered to have a low risk of bias, articles investigating a relationship between tendon regeneration and determinants of regeneration or clinical outcome had to use valid determinants as well as an unbiased assessment of the study outcome and

determinants. Two independent researchers performed the risk-of-bias assessment. Disagreement was solved by consensus.

Table 1. Criteria for the risk-of-bias assessment.

Question	Response
1. Is there a clearly stated aim?	The study must have a study question, main aim or objective. The question addressed must be precise and relevant in light of the available literature. To be judged as *adequate*, the aim of the study must be consistent with the description given in the introduction of the paper.
2. Were consecutive patients included?[a, b]	The investigators must state 'consecutive patients' or 'all patients during period from *X* to *X*.'
3. Are inclusion and exclusion criteria described?	Inclusion and exclusion criteria must be reported.
4. Is the inclusion of patients described?	The number of eligible patients who agreed to participate (ie. gave consent) must be reported.
5. Was data collection prospective? That is, were data collected according to a protocol established before the beginning of the study?	The investigators should state 'prospective' or 'follow-up'. A study is not prospective when the study design is a chart review or database review.
6. Was the imaging technique used to confirm regeneration valid and reliable?[a, b]	To be judged as *adequate*, at least 1 of the following imaging techniques must be used: histological biopsy, magnetic resonance imaging, echo / ultrasound, computed tomography. All other imaging techniques are judged as inadequate.
7. Was assessment of the study outcome and determinants unbiased?[b]	To be judged as *adequate*, outcome(s) and determinants have to be measured independently of each other.
8. Were the determinants measures used accurate (valid and reliable)?[b]	To be judged as *adequate*, the determinant measures must be shown to be valid and reliable, or the investigators must refer to other work that demonstrates the determinant measures to be accurate.
9. Was the follow-up period appropriate for the aim of the study?	To be judged as *adequate,* the study must report the follow-up period, and a study must entail 3 months' minimal follow-up.
10. Was loss of follow-up reported and acceptable?	To be judged as *adequate*, the study must report the loss of follow-up, and the loss of follow-up must be ≥20%.
11. Was the sample size calculated before the study was initiated?	To be judged as *adequate,* calculation of the sample size must have been made before the study was initiated.
12. Were the statistical analyses adequate?	To be judged as *adequate,* the following aspects must be met: - The relationship between the determinant and the primary outcome was described. - There was an adjustment for age and/or sex. A study was inadequate if the effect of the main confounders was not investigated or confounding was demonstrated but no adjustment was made in the final analyses. - The variance of the outcome was reported (e.g. standard deviation, confidence interval)

[a] *Judged as adequate for studies investigating the rate of hamstring tendon regeneration.*
[b] *Judged as adequate for studies investigating a relationship between hamstring tendon regeneration and determinants or clinical outcome.*

When the article met the criterion, 1 point was granted, if the criterion was not met, 0 points were given. If the information concerning the specific criterion was not available in the study and information was not available after contacting the authors, 0 points were given.

Statistical analysis

In this systematic review, data for hamstring tendon regeneration were pooled. Regeneration rates less than 1 year after harvesting were pooled, and regeneration rates more than 1 year after harvesting were pooled. Distribution of the pooled data are displayed as median and interquartile range (IQR).

RESULTS

Literature search

From initial 2957 relevant articles identified, 2939 publications were excluded based on title and abstract, because they did not meet the inclusion criteria. Consequently, a total of 18 studies were included. A flow chart of the literature search is presented in Figure 1. Hamstring tendon regeneration rates were reported in 17 of the included studies, and 6 of the included studies reported possible determinants for hamstring regeneration or clinical outcome.

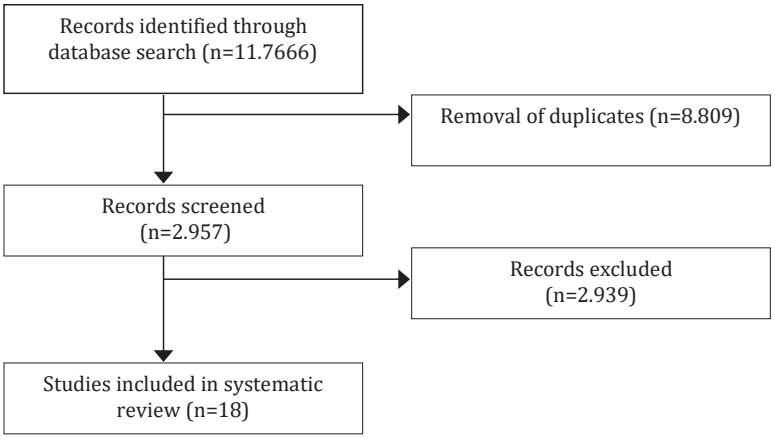

Figure 1. Flow chart.

Risk-of-bias assessment

According to the predefined criteria, 6 articles that considered the rate of hamstring tendon regeneration had a low risk of bias[4, 11, 15, 26, 27, 30]. Three studies investigating

possible determinants for hamstring regeneration or clinical outcome had a low risk of bias[4, 11, 26] (Supplementary Table 2). Other studies did not meet the criteria and were therefore considered to have a high risk of bias.

Study characteristics

The study sizes ranged from 10 to 50 patients. The average age of the included patients varied from 20 to 37 years. Male participation ranged from 27% to 100%. Follow-up time ranged from 1 week to 10 years. Table 2 shows the data extraction of the studies evaluating hamstring tendon regeneration after harvesting.

Measuring methods

The included studies used different imaging techniques to determine regeneration of the hamstring tendons. Magnetic Resonance Imaging (MRI) was the most common used technique (12/18)[2, 4, 9, 11, 15, 19, 23, 24, 28, 30, 34, 35]. Other techniques used were 3-dimensional computed tomography (2/18)[20, 21], histological biopsy (3/18)[9, 26, 31] and ultrasound (3/18)[3, 27, 31].

To be assessed as regeneration, all the included studies demanded at least regrowth of tendon tissue. Next to this, different studies used their own scoring system with additional points of interest (e.g. cross-sectional area of muscles and tendons, muscle volume, muscle length, proximal shift of the musculotendinous junction, pixel value, and insertion site) to assess the presence or absence of regeneration.

Tendon regeneration

All included studies reported their exact regeneration rates except from Rispoli et al[28]. The regeneration rates varied overall from 50% to 100% for the semitendinosus tendon and from 46% to 100% for the gracilis tendon (Table 3). Regeneration of the gracilis tendon was only measured by use of MRI. After the data were pooled, the overall mean regeneration rate in the first year after harvesting was 91% (median [IQR], 97[74-100]) for the semitendinosus and 100% for the gracilis tendon. The overall mean regeneration rate more than 1 year after harvesting was 79% (median [IQR], 80 [75.5-90]) for the semitendinosus and 72% (median [IQR], 80 [61-88.5]) for the gracilis.

Time path of tendon regeneration

Five studies determined the regeneration rate at different points in the first year after ACL reconstruction. Eriksson et al.[11] described that no tendon regeneration could be observed 2 weeks after surgery, but 6 months after surgery, the majority of the patients (73%) showed regeneration.

Table 2. Data extraction.

		Study participants					
Author (Year)	**Study design**	**No.**	**Sex, % male**	**Age at start study, y, (mean or median (± SD[b])**	**Follow-up time, mo (mean or median (± SD[b])**	**Imaging technique**	**Experience examiner (No. of examiners)**
Eriksson et al.[11](1999)	Prospective study	11	73	24	6-12	MRI	MRI radiologist (1)
Papandrea et al.[27] (2000)	Prospective study	40	73	28	24	US	Orthopaedic surgeon (1)
Eriksson et al.[9] (2001)	Case series	16	88	26	MRI, median: 7 Histology, median: 10	MRI / Histology	MRI: MRI radiologist (1) Histology: unknown
Rispoli et al.[28] (2001)	Case series	20	65	37	32	MRI	Musculoskeletal radiologist (2)
Tadokoro et al.[34] (2004)	Retrospective study	28	36	22	67.2	MRI	NR
Nakamae et al.[21](2005)	Prospective study	29	52	28	12	3D-CT	Orthopaedic surgeon (2)
Nishino et al.[23] (2006)	Prospective study	23	43	22 (±4)	23	MRI	NR
Okahashi et al.[26] (2006)	Prospective study	11	27	23	12	Histology	Orthopaedic surgeon (1)
Takeda et al.[35] (2006)	Prospective study	11	55	21	12.7	MRI	NR
Ahldén et al.[1] (2012)	Case series	19	53	Median: 23	Median: 102	MRI	Musculoskeletal radiologist(1)
Bedi et al.[3] (2012)	Case series	15	40	27	96.3	US	Musculoskeletal radiologist (1)
Choi et al.[4] (2012)	Case series	45	100	33 (±7)	36.4 (±7.4)	MRI	Musculoskeletal radiologist (1)
Janssen et al.[15] (2012)	Prospective study	22	77	28 (±5)	12	MRI	Orthopaedic surgeon (1) and Radiologist (1)
Murakami et al.[19] (2012)	Prospective study	20	55	23*	15	MRI	Orthopaedic surgeon (3)
Nakamae et al.[20] (2012)	Retrospective study	39	56	Group 1: 30 (±12) Group 2: 27.1 (±11.4)	6 and/or 12	3D-CT	Orthopaedic surgeon (2)
Snow et al.[30] (2012)	Retrospective study	10	70	33	129	MRI	Orthopaedic surgeon (2)
Stevanović et al.[31] (2013)	Prospective study	50	70	25 (±4)	US: 24 Histology: unknown	US / histology	NR
Nomura et al.[24] (2014)	Prosepective study	24	58	21 (±2)	28 ± 18	MRI	Orthopaedic surgeon (1)

[a] *3D-CT, three-dimensional computed tomography; MRI, magnetic resonance imaging; US, ultrasound; NR, not reported.*

[b] *Standard deviation given if reported in the original study.*

Table 3. Regeneration rates before and after 1 year of follow-up.

		Regeneration rate, % (n/N)			
		≤1-y follow-up		>1-y follow-up	
Author (Year)	**Imaging technique**	**Semitendinosus**	**Gracilis**	**Semitendinosus**	**Gracilis**
Eriksson et al.[11] (1999)	MRI	73 (8/11)			
Papandrea et al.[27] (2000)	US	100 (40/40)			
Eriksson et al.[9] (2001)	MRI/ Histology	75 (12/16)			
Rispoli et al.[28] (2001)	MRI	100 (20/20)			
Tadokoro et al.[34] (2004)	MRI			79 (22/28)	46 (13/28)
Nakamae et al.[21] (2005)	3D-CT	100 (20/20)			
Nishino et al.[23] (2006)	MRI			91 (21/23)	
Okahashi et al.[26] (2006)	Histology	82 (9/11)			
Takeda et al.[35] (2006)	MRI			100 (11/11)	82 (9/11)
Åhldén et al.[1] (2012)	MRI			89 (17/19)	95 (18/19)
Bedi et al.[3] (2012)	US			50 (9/18)	
Choi et al.[4] (2012)	MRI			80 (36/45)	76 (34/45)
Janssen et al.[15] (2012)	MRI	64 (14/22)	100 (22/22)		
Murakami et al.[19] (2012)	MRI	100 (16/16)			
Nakamae et al.[20] (2012)	3D-CT	97 (38/39)			
Snow et al.[30] (2012)	MRI			80% (8/10)	
Stevanović et al.[31] (2013)	US/ Histology			72 (18/25)	
Nomura et al.[24] (2014)	MRI			88 (21/24)	
Total		91 (177/195)	100 (22/22)	79 (142/179)	72 (74/103)
Median (Interquartile range)		97 (74-100)		80 (75.5-90)	80 (61-88.5)

[a]*Data are reported as percentage (absolute values) unless otherwhise indicated. 3D-CT, three-dimensional computed tomography; MRI, magnetic resonance imaging; US, ultrasound.*

Nakamae et al.[21] reported that no regeneration could be observed 1 month after surgery. However, 90% of the patients showed regeneration at 9 months after ACL reconstruction, and all the patients showed regeneration after 1 year[21].

In accordance with Eriksson et al.[11], Papandrea et al.[27] did not report any regeneration after 2 weeks. Papandrea et al.[27] reported that after 12 months, all fibers of the regenerated tendon were attached to the medial popliteal fascia.

Rispoli et al.[28] made no differentiation between regeneration of the semitendinosus and gracilis tendon, but the authors reported fluid or edema in the semitendinosus and gracilis tract 2 weeks after harvesting. Although a neotendon seemed to be present after 12 months, the most distal 3 to 4 cm of this neotendon remained ill defined[28].

Murakami et al.[19] used an inducer technique meaning that the gastrocnemius branch of the harvested semitendinosus was used as a graft to improve the regeneration process. This study reported tendon regeneration in all patients 1 month after ACL reconstruction.

These five studies show that the process of regeneration took place the first year after harvesting and that the regeneration rate could be 100% after one year. However, none of these studies reported a clearly defined time period of regeneration. Other studies, with only one evaluation moment, reported regeneration rates for the semitendinosus ranging from 64% to 97%[15, 20].

Determinants for tendon regeneration

Six publications reported possible determinants, such as sex, demographic data, and duration of immobilization[4, 9, 11, 20, 26, 35].

Patient sex Only 5 publications made a distinction in regeneration rated based on sex[4, 9, 11, 26, 35]. In these publications collectively, regeneration in men could be observed in 85.5% of the cases and in women in 83.3% of the cases. No study reported a significant difference in regeneration rate between men and women.

Demographic data Choi et al.[4] and Nakamae et al.[20] investigated the effect of several demographic factors on hamstring tendon regeneration. No significant difference in hamstring tendon regeneration could be found based on age, weight, or height.

Duration of immobilization Nakamae et al. described the effect of duration of immobilization after ACL reconstruction on tendon regeneration. They divided the study population into 2 groups: a control group with a standard rehabilitation protocol with 3 days of immobilization (short immobilization) and the intervention group with of 10 to 14 days of immobilization (long immobilization). In the short immobilization group, all patients but one showed tendon regeneration. In the long immobilization group, a tendon-like structure was confirmed in all cases. The difference in regeneration rate was not statistically significant ($p=0.42$)[20].

Tendon regeneration in relationship with clinical outcome

Seven studies determined whether tendon regeneration influenced the clinical outcome[4, 9, 15, 19, 20, 23, 34]. Clinical outcome was defined as hamstring function and hamstring strength.

Choi et al.[4] noted that patients without regenerated tendons had more than 4 times as much flexor strength deficit compared with patients with 2 regenerated tendons ($p<0.05$). Furthermore, a correlation ($\rho=-0.443$) was noted between the number of regenerated tendons and the amount of functional deficit. This contradicts the results of Janssen et al.[15] who did not report a significant difference in flexion and extension strength between the patients with both hamstring tendons regenerated and the patients with 1 regenerated tendon.

Eriksson et al.[9] performed several functional performance tests. The Lysholm scores showed no statistical difference between the regeneration and no-regeneration group.

Furthermore, regarding hamstring strength, no statistical difference between the regenerated group and non-regenerated group could be found.
Nakamae et al.[20] considered hamstring strength and reported no significant correlation between hamstring peak torque and the types of regenerated tendon.
Nishino et al.[23] showed that hamstring strength was greatest when the semitendinosus tendon regenerated and had a normal length. Hamstring strength was lowest when no semintendinosus tendon-like structure could be identified. Unfortunately, no p-values were reported.
Using ultrasound, Tadokoro et al.[34] were able to differentiate between different morphologic regeneration (hypertrophic, atrophic, and unidentifiable regeneration) of semitendinosus and gracilis tendons. The hamstring strength of the operated leg was compared with the hamstring strength in the nonoperated side. The nonoperated side had significantly greater hamstring strength in all cases, except for the hypertrophic gracilis tendon group (p=0.077).

DISCUSSION

This systematic review aimed to provide an overview of the current evidence regarding hamstring tendon regeneration after harvesting.
The mean regeneration rate less than 1 year and at least 1 year after harvesting for the semitendinosus tendon was 91% (median [IQR], 97 [74-100]) and 79% (median [IQR], 80 [75.5-90]), respectively; for the gracilis tendon, it was 100% and 72% (median [IQR], 80 [61-88.5]), respectively. The majority of the hamstring tendon regeneration was found to occur between 1 month and 1 year after harvest. No determinants for tendon regeneration are described. Six studies determined whether tendon regeneration influenced the clinical outcome. However, results of these studies are contradictory.
The included studies reported a wide range of regeneration rates. Several explanations can be found for this variation. First, all the included studies used other points of interest to assess the rate of regeneration. Second, the assessments are mostly dichotomous, which is not in accordance with a gradual, continuous process expected in tendon regeneration. Third, studies used different imaging techniques to visualize tendon regeneration. It is unlikely that these techniques are equal in all aspects to determine the hamstring regeneration. Fourth, patient characteristics such as sample size, age, and sex differed. In short, the wide range in reported regeneration rates might be due to the heterogeneity in study designs and how tendon regeneration was assessed.
We found counterintuitive results when comparing the high regeneration rates less than 1 year after harvesting and the relatively low regeneration rates more than 1 year after harvesting. Our aim is to identify the time course of regeneration. This could be

established best if only prospective studies were included, measuring regeneration rates at different points in time. Studies measuring regeneration only once are less accurate, as it is unknown whether regeneration was present before. Considering the included studies in this systematic review, it becomes clear that a majority of the studies reporting regeneration rates in the first year only had 1 measurement moment[9, 19, 21, 27, 28]. This may have contributed to an overestimation in studies measuring regeneration rates less than 1 year after harvesting.

The current systematic review aims to clarify the time course of regeneration. Janssen and Scheffler[14] described in a systematic review 3 different stages of a regenerating hamstring; however, the time course of these stages remained unclear. Five studies assessed the regeneration rates in patients at different chronological moments the first year after harvesting for ACL reconstruction[11, 19, 21, 27, 28]. Four of these studies reported a regeneration rate of 100% after one year[19, 21, 27, 28]. This result was contradictory to studies that used one measure point in time, as several studies reported regeneration rates less than 100% in the first year after surgery. Therefore, it remains unclear when regeneration is completed and whether reported regeneration rates in the first year after harvesting are an overestimation or an underestimation, respectively, due to studies with several measurement moments and with a single measurement moment. Studies that used more than 1 evaluation point measured a different number of patients at each evaluation point. It was not reported whether these patients were the same individuals as the ones who were evaluated before[11, 21, 28]. So the exact time course of regeneration could not be exactly clarified, but the majority of hamstring tendon regeneration was found to occur between 1 month and 1 year after harvest.

Another aim of this systematic review was to identify predictive factors for regeneration. Some studies mentioned regeneration rates in men and women separately, but sex as a determinant for hamstring tendon regeneration has never been researched. Vourazeris et al. considered the possibility of fatty infiltration as an inhibiting factor for tendon regeneration in rabbits. However, no fatty infiltration could be found over time after hamstring tendon harvesting[36]. Fatty infiltration cannot be considered as a determinant. Altogether, we conclude that neither positive nor negative predictors for hamstring tendon regeneration have been described in current literature.

Only 7 studies investigated the relationship between regeneration and clinical outcome[4, 9, 15, 19, 20, 23, 34]. However, these results were contradictory. Choi et al.[4] reported that the number of regenerated tendons influenced hamstring function. Thus, the clinical consequences of the absence of regeneration remain unclear.

In future, more research is required to identify determinants of hamstring tendon regeneration. This is important, because if any determinants can be specified, a risk profile for regeneration failure could be developed. Based on this risk profile, it will be possible to assess whether reharvesting may be possible in the future. Further, more knowledge

about the clinical outcome in terms of hamstring strength and hamstring function after regeneration may influence the type of surgery chosen. However, because the clinical consequences of absence of regeneration remain unclear, better studies are needed to clarify this. Rehabilitation programs should be redesigned if it is found that mechanical load is a positive or negative predictive factor for regeneration. Further, knowledge about the time course of regeneration can change rehabilitation programs, because without hamstring regeneration these muscles cannot be rehabilitated or exercised.

The risk-of-bias assessment that we performed showed that the probability of bias is high. Six studies that examined hamstring tendon regeneration were considered to have a low risk of bias[4, 11, 15, 26, 27, 30]. Only Choi et al.[4], Eriksson et al.[11], and Okahashi et al.[26], investigating the relationship between hamstring tendon regeneration and determinants of regeneration and clinical outcome, met the criteria described in the methods section[4, 11, 26]. The strength of evidence is therefore limited because of the quality of the available studies. Another weakness of this systematic review is the population size in the included studies. Only 2 studies performed a calculation of sample size, and other studies were underpowered to allow firm conclusions. However, this systematic review pooled data concerning hamstring regeneration and therefore approximated real regeneration rates. For this reason, we conclude that hamstring tendons regenerate after harvesting in at least 70% of the cases.

In conclusion, the results of this systematic review indicate that the semitendinosus and gracilis tendon regenerate in the majority of the patients after harvesting for ACL reconstruction. The pooled regeneration rate for the semitendinosus tendon and for the gracilis tendon is at least 70% in all cases. While the exact time couse of regeneration could not be determined exactly due to heterogeneity of the study designs, the majority of hamstring tendon regeneration was found to occur between 1 month and 1 year after harvest. No positive or negative determinants for tendon regeneration have been described yet. Because of conflicting evidence, no correlation could be described between tendon regeneration and clinical outcome. Considering the possible potential clinical effect, it is of vital importance to perform more prospective research concerning hamstring tendon regeneration after harvesting, its functional deficit, and determinants that influence regeneration.

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SUPPLEMENTARY DATA

Supplementary Table 1. Search terms.

(Hamstring/de OR 'semitendinous muscle'/de OR 'gracilis muscle'/de OR (hamstring* OR semitendin* OR gracilis* OR ((single OR double) NEAR/3 bundle*)):ab,ti) AND (harvesting/de OR autograft/de OR 'tendon graft'/de OR 'anterior cruciate ligament reconstruction'/de OR 'anterior cruciate ligament'/de/dm_su OR 'anterior cruciate ligament injury'/de/dm_su OR 'anterior cruciate ligament rupture'/de/dm_su OR (('anterior cruciate ligament'/de) AND ('ligament surgery'/de)) OR (harvest* OR autograft* OR autotransplant* OR gathering* OR transect* OR ((acl OR 'anterior cruciate') NEAR/3 (surg* OR repair* OR reconstruct*))):ab,ti) AND (regeneration/exp OR evaluation/de OR 'muscle function'/de OR strength/de OR 'muscle strength'/de OR 'tensile strength'/de OR torque/de OR 'knee function'/de OR 'neuromuscular function'/de OR 'range of motion'/de OR 'muscle contraction'/de OR 'physical examination'/de OR 'medical examination'/exp OR 'function test'/de OR 'joint laxity'/de OR 'knee instability'/de OR 'joint instability'/de OR biomechanics/de OR (recover* OR regenerat* OR evaluat* OR function* OR strength* OR torque* OR torsion* OR force* OR flexion* OR (range NEAR/3 motion*) OR (physical* NEAR/3 examin*) OR stabilit* OR instab* OR laxit* OR rotat* OR biomechanic*):ab,ti) NOT ([animals]/lim NOT [humans]/lim) AND ([english]/lim OR [dutch]/lim) NOT ([meta analysis]/lim OR [systematic review]/lim OR [editorial]/lim OR [letter]/lim OR [note]/lim OR [review]/lim)

SUPPLEMENTARY DATA

Supplementary Table 2. Risk-of-bias assessment.

Author (Year)	1	2[a, b]	3	4	5	6[a, b]	7[b]	8[b]	9	10	11	12	Risk of bias
Eriksson et al.(1999)	1	1	0	0	1	1	1	1	0	0	0	0	Low
Papandrea et al. (2000)	1	1	0	0	1	1	0	0	1	0	0	0	Low
Eriksson et al. (2001)	1	0	1	0	1	1	1	1	1	1	0	0	High
Rispoli et al. (2001)	1	0	0	1	1	1	0	0	1	0	0	0	High
Tadokoro et al. (2004)	1	0	1	0	0	1	0	0	1	0	0	0	High
Nakamae et al.(2005)	1	0	1	0	1	1	0	0	1	0	0	0	High
Nishino et al. (2006)	1	0	0	0	1	1	0	0	1	0	0	0	High
Okahashi et al. (2006)	0	1	0	1	1	1	1	1	1	0	0	0	Low
Takeda et al. (2006)	1	0	0	0	1	1	0	1	1	0	0	0	High
Ahlen et al. (2012)	1	0	0	0	0	1	0	0	1	0	1	0	High
Bedi et al. (2012)	1	0	1	0	1	1	0	0	0	0	0	0	High
Choi et al. (2012)	1	1	1	0	1	1	1	1	1	0	0	1	Low
Janssen et al. (2012)	1	1	1	0	1	1	0	0	1	0	1	0	Low
Murakami et al. (2012)	1	0	0	0	1	1	0	0	1	0	0	0	High
Nakamae et al. (2012)	1	0	1	0	0	1	0	0	1	0	1	0	High
Snow et al. (2012)	1	1	1	0	1	1	0	0	1	0	0	0	Low
Stevanovic et al.(2013)	1	0	1	1	1	1	0	0	1	0	0	0	High
Nomura et al. (2014)	1	0	1	0	1	1	0	0	1	0	1	0	High

The numbers 1 to 12 represent questions from the risk of bias assessment.
[a]studies reporting about hamstring tendon regeneration rate should obtain 1 point to decrease the risk of bias.
[b]studies investigating relationship between tendon regeneration and determinants should obtain 1 point to decrease the risk of bias.

CHAPTER 3

Remodeling of regenerated hamstring tendons: a magnetic resonance imaging study

Under review

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ABSTRACT

Background: Patients often report pain in the posterior thigh following harvest of the hamstring tendons. This is potentially caused by impaired regeneration of the tendons or altered morphological features of the regenerated structures. Therefore, this study aims to describe the regeneration and remodeling process of the hamstring tendons on magnetic resonance (MR) imaging.

Methods: Patients with anterior cruciate ligament (ACL) injury who underwent reconstruction using the hamstring tendons were included in the current study. MR imaging was preoperatively acquired and at 1- and 2-year follow-up after surgery. Hamstring tendon regeneration and sizes were evaluated at knee joint line level.

Results: 76 out of 93 patients had sagittal and transversal MR images available at 1- and 2-year follow-up. Two years after surgery, semitendinosus (ST) tendons regenerated in 65.8% and gracilis (G) tendons in 82.9%. At 2-years follow-up 10.5% of the patients showed an altered regeneration status compared to the first year after surgery. The sizes of native ST tendons (mean, interquartile range [IQR], 11.6 mm^2 [9.1-13.3]) and gracilis tendons (mean [IQR], 7.3 mm^2, [6.0-8.5]) significantly increased 2 years after surgery to 22.7 mm^2 (IQR 11.2-24.4, p=0.02) and 13.6 mm^2 (IQR 7.8-18.4, p=0.01) respectively. Additionally, musculotendinous junctions shifted proximally in 57.1% of the ST and in 78.6% of the G tendons.

Conclusions: The regeneration status of ST and G tendons changed in 10.5% of the patients over time, resulting in 65.8% and 82.9% respectively at 2-year follow-up. Regenerated tendons are hypertrophic and longer compared to their native ones.

Key words: Anterior Cruciate Ligament Reconstruction; Regenerative Medicine; Translational Research; Hamstring Tendons; Sports Medicine.

INTRODUCTION

Hamstring tendon autografts are widely used to anatomically reconstruct a variety of structures, such as the anterior cruciate ligament (ACL), the medial patellofemoral ligament, lateral ankle ligaments and the coracoclavicular ligament. More specifically, the semitendinosus (ST) tendon and/or gracilis (G) tendon are harvested for these reconstruction procedures. A particularly interesting feature of hamstring tendons is their potential to regenerate after harvest, which is observed in at least 70% of the patients[15]. This regeneration process of the hamstring tendons is less likely to occur with aging and in smokers[16]. Although hamstring tendon regeneration has been investigated extensively, knowledge about the tendon remodeling process is limited.

Hamstring tendon remodeling should be considered as a dynamic and continuous process that changes morphologic characteristics of the tendons over time, such as the cross-sectional area (CSA) and tendon lengths. In addition, impaired or delayed tendon remodeling might influence regeneration rates at different follow-up periods. However, the vast majority of the current literature assesses hamstring tendon regeneration dichotomously and only uses a single follow-up period[15]. Therefore, the process of tendon remodeling following tendon regeneration remains unclear.

Knowledge about hamstring tendon remodeling is of clinical importance for several reasons. First, impaired tendon remodeling and premature rupture of the regenerated structure might cause retraction of the muscle belly, resulting in clinical symptoms such as posterior thigh pain, weakness and cramping[7]. Second, many patients voice concerns about the resection of functional tendons and possibly accompanying functional deficits, potentially caused by impaired remodeling mechanisms. Although it has been suggested before that regenerated tendons could be used as grafts[14, 21], this might partially depend on the quality of the tendon remodeling process.

The current magnetic resonance (MR) study aims to describe the hamstring tendon remodeling process. In order to assess the remodeling process, the current study focuses on three specific outcomes that were repeated at 1-and 2-year follow-up. First of all, impaired or delayed remodeling potentially influences hamstring tendon regeneration rates. Therefore, hamstring tendon regeneration rates were measured at both 1- and 2-year follow-up to assess the quality of the remodeling process. Additionally, the remodeling process is likely to influence morphologic features of tendons, such as CSAs and tendon lengths.

METHODS

Study population

The patients were recruited between January 2009 and November 2010 in a prospective multicenter follow-up study from three hospitals in The Netherlands: Erasmus MC – University Medical Center Rotterdam, Medical Center Haaglanden (The Hague) and Reinier de Graaf Gasthuis (Delft)[18]. In the current study, we included patients that participated in the KNALL (KNee osteoarthritis anterior cruciate Ligament Lesion) study and underwent a surgical ACL reconstruction entailing both the ST and G tendons. Inclusion criteria for this study were (1) ACL rupture diagnosed by physical examination and MRI, (2) MRI was preoperatively acquired within 6 months after trauma, (3) patients were between 18 and 45 years old. Patients who did not speak Dutch, those with previous ACL injury or intra-articular knee trauma or surgery, those with disabling co-morbidity and those with osteoarthritic changes on radiography (Kellgren and Lawrence grade > 0) were excluded. Written informed consent was obtained from all included patients and the institutions' Medical Ethics Committees approved the study (NL 21778.078.08, MEC-2008-068).

MRI measurements

MR examinations were performed before surgical reconstruction (baseline), at 1-and 2-year follow-up. At baseline, MR scans were acquired using three different MR scanners (Philips, Siemens or General Electric). The follow-up MR scans were acquired on the same MR scanner at 1.5 Tesla. Patients' knees were imaged in a neutral position using a dedicated knee coil. Included MR scans have the following MR pulse sequences: sagittal and coronal proton density weighted turbo spin echo (TSE) sequence (slice thickness 3 mm, TR/TE: 2700/27ms), coronal T2-weighted TSE sequence with fat saturation (slice thickness 3 mm, TR/TE: 5030/71 ms), axial proton density and T2-weighted TSE sequence (slice thickness 3 mm, TR/TE: 3500/25/74ms) and sagittal 3D water excitation double-echo steady state (slice thickness 1.5 mm, TR/TE 21.35/7.97ms).

Assessment of regenerated tendons on MRI

To assess presence of regenerated hamstring tendons, both axial and sagittal MR planes at 1- and 2-year follow-up had to be available. Based on previous findings, hamstring tendon regeneration in the current study was subdivided into three different categories: complete, incomplete or no regeneration[15]. If regenerated ST and G tendons could be visualized at the level of the joint line on axial and sagittal planes MR images, the regeneration was considered as complete. Tendon regeneration was considered as incomplete when a tendon-like structure was absent at the joint line level, but could be identified cranially

thereof. If no neotendon could be visualized on any MR image on any level, this was considered as no regeneration.

The cross-sectional area (CSA) of the hamstring tendons was assessed in patients with complete regeneration of both the ST and G tendons and with MR imaging available at baseline (native tendon), 1- and 2-year follow-up (regenerated tendons). CSAs were measured in the axial plane at the joint line level using a commercially available MR image analysis software (AW Server 2.0, GE Health care).

The location of the musculotendinous junction of the ST and G was determined on axial MR images as the most caudal image on which tissue with muscle signal intensity was visualized. This anatomic position was then co-localized on the sagittal plane and the distance between this location and the extension of the joint line was measured. Musculotendinous junctions could be determined in patients with complete regeneration of both hamstring tendons and with MR imaging visualizing the distal musculotendinous junction at all three time points.

All MR measurements were performed by a trained researcher (M.S.) under supervision of a musculoskeletal radiologist (E.O.) and a sports medicine trained orthopaedic surgeon (D.M.) with both more than ten years of experience. Baseline and follow-up MR scans were assessed concurrently and the order of MR measurements was known. Equivocal cases were discussed and solved by consensus.

Statistical analysis

All statistical analyses were performed with IBM SPSS Statistics for Windows (version 21.0, IBM Corp., Armonk, NY). To test for normality, a Shapiro-Wilk's test ($p>0.05$) and inspection of the histograms, normal Q-Q plots and box plots were performed. Interquartile range (IQR) was obtained for non-normally distributed variables. Furthermore, data was tested on skewedness and kurtosis. To determine the interobserver variability 20 randomly chosen scans were re-assessed by a blinded second observer (E.O.), and an inter- and intraclass correlation coefficient (two-way random effects model, absolute agreement) was calculated.

RESULTS

Study population

A flow chart of selection of eligible patients is shown in Figure 1. 93 patients met the inclusion criteria for the current study. Axial and sagittal MRI planes of 76 patients at both post-operative follow-ups were available for analysis. Baseline patient characteristics are presented in Table 1. Mean age at trauma was 25.8 years (SD 6.6) and 65.3% were men.

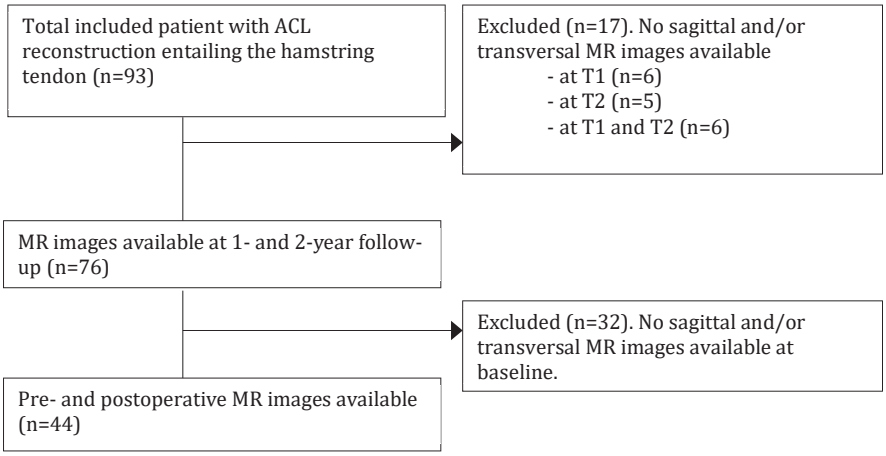

Figure 1. Overview of included patients.
[a]*ACL, Anterior Cruciate Ligament; MR, Magnetic Resonance; T1, 1-year follow-up; T2, 2-year follow-up.*

Table 1. Patient characteristics[a].

	n=76
Age at trauma, y	25.8 ± 6.6
Sex (male) – n (%)	49 (65.3)
Body mass index, kg/m^2	24.2 ± 3.3
Time from surgery to MRI at 1-year follow-up, m	9.5 ± 2.7
Time from surgery to MRI at 2-year follow-up, m	21.1 ± 4.2

[a]*Data are presented as mean ± SD unless otherwise specified.*

Regeneration rates

At 1-year follow-up after harvesting of the hamstring tendons, 43.3% of the patients showed complete regeneration of both tendons. On the contrary, 10.5% of the patients showed no regeneration at all. Complete regeneration of the ST tendon was visualized in 53.9% of the patients, whereas incomplete regeneration was found in 17.1% of the patients. No signs of ST regeneration were found in 28.9% of the patients. The G tendon regenerated in 59.2% of the patients and showed incomplete regeneration in 25.0% of the patients. In 10 patients, complete regeneration of one tendon was accompanied with no regeneration of the other tendon. Regeneration rates 9.5 months after harvest are displayed in Table 2A.

At 2-year follow-up, both tendons regenerated in 42.1% and 11.8% of all patients had no tendon regeneration (Table 2B). Complete regeneration of the ST tendon took place in 53.9% of the patients two years after surgery and incomplete regeneration was found in 11.8%. At 2-year follow-up, the G tendon regenerated completely in 59.2% of the patients and did not regenerate in 17.1% of the patients.

Interestingly, the regeneration status changed in 8 patients (10.5%) over time. The regeneration status of the semitendinosus tendon deteriorated in 50% (4 out of 8), whereas the gracilis tendon deteriorated in only 12.5% (1 out of 8) of the patients. The regeneration status of the semitendinosus and gracilis improved in 37.5% (3 out of 8) and 25% (2 out of 8) respectively at 2-year follow-up. This implies that 6.7% (5 out 76) of the patients improved their regenerated structure over time (Table 3).

At 1-year follow-up, the regeneration starting point was in all 33 cases of incomplete tendon regeneration found at the distal muscle sites. This was confirmed in all 28 cases of incomplete regeneration at 2-year follow-up. No concurrent regeneration sites could be identified.

Table 2. Regeneration rates.

A. Regeneration at 1-year follow-up.

		Semitendinosus			
		Complete	Incomplete	No	
Gracilis	Complete	33 (43.4)	5 (6.6)	7 (9.2)	45 (59.2)
	Incomplete	5 (6.6)	7 (9.2)	7 (9.2)	19 (25.0)
	No	3 (3.9)	1 (1.3)	8 (10.5)	12 (15.8)
		41 (53.9)	13 (17.1)	22 (28.9)	76 (100.0)

n (%).

B. Regeneration 2-year follow-up.

		Semitendinosus			
		Complete	Incomplete	No	
Gracilis	Complete	32 (42.1)	3 (3.9)	10 (13.2)	45 (59.2)
	Incomplete	6 (7.9)	5 (6.6)	7(9.2)	18 (23.7)
	No	3 (3.9)	1 (1.3)	9 (11.8)	13 (17.1)
		41 (53.9)	9 (11.8)	26 (34.2)	76 (100.0)

n (%).

Table 3. Patients with altered regeneration status[a].

Patient number	1-year follow-up		2-year follow-up		Change in regeneration status	
	ST	G	ST	G	ST	G
1	+	+	+	±	=	↓
2	+	+	-	+	↓	=
3	+	+	-	-	↓	↓
4	+	±	-	+	↓	↑
5	±	+	-	+	↓	=
6	±	+	+	+	↑	=
7	±	±	+	±	↑	=
8	±	±	+	+	↑	↑

[a]ST, semitendinosus; G, gracilis; +, complete regeneration; ±, incomplete regeneration; -, no regeneration, ↑ improvement of regeneration status; ↓ deterioration of regeneration status; =, no change in regeneration status.

Cross-sectional areas

Of the 30 patients with complete regeneration of both tendons, a total of 15 patients had MR imaging available at baseline, 1- and 2-year follow-up. Representative images are displayed in Figure 2A-C.

The mean CSA of the native ST tendons was 11.6 mm^2 (IQR 9.1-13.3), whereas 9.5 months after tendon harvesting the mean CSA of regenerated ST tendons was increased to 25.1 mm^2 (IQR 15.0-27.0) (p=0.04). Compared to the native tendon, the mean CSA of the ST tendons increased to 22.7 mm^2 (IQR 11.2-24.4, p=0.02) at 2-year follow-up (Figure 3A). The average CSA of native G tendons was 7.3 mm^2 (IQR 6.0-8.5). This CSA increased to an average of 17.5 mm^2 (IQR 11.2-21.5, p<0.01) 9.5 months after surgery and to 13.6 mm^2 (IQR 7.8-18.4, p=0.01) at 2-year follow-up (Figure 3B).

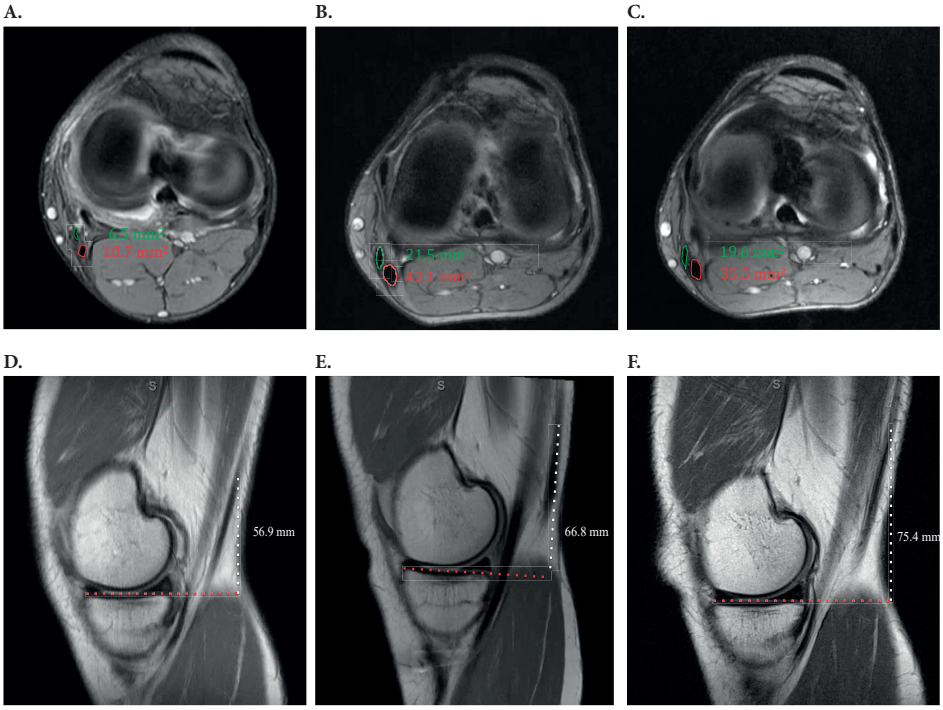

Figure 2. Representative MR images of same patient.

***A-C**: Images of the cross-sectional area of the left knee before surgery (A), at one year follow-up (B), and 2-year follow-up (C). Red color indicates semitendinosus tendon, green color indicates gracilis tendon.*

***D-F**: images of the musculotendinous junction of the semitendinosus tendon in a single patient A) before harvesting, B) at 1-year follow-up, C) at 2-year follow-up. Red dotted line indicates extension of the joint line, white dotted line indicates distance between musculotendinous junction and extension of joint line.*

Tendon lengths

Of the 30 patients with complete regeneration of both tendons, musculotendinous junctions of the ST and G could be visualized at all follow-up measurements in 8 and 14 patients, respectively. A shift in the location of musculotendinous junction of the ST could be determined in 8 patients: the junction was found to be located more proximally at both post-operative time points compared to that of the native ST tendons. Similarly, of 14 patients in whom the musculotendinous junction of the G tendon could be visualized pre-operatively, a proximal shift occurred in 11 patients at both post-operative time-points. Representative images are displayed in Figure 2D-F.

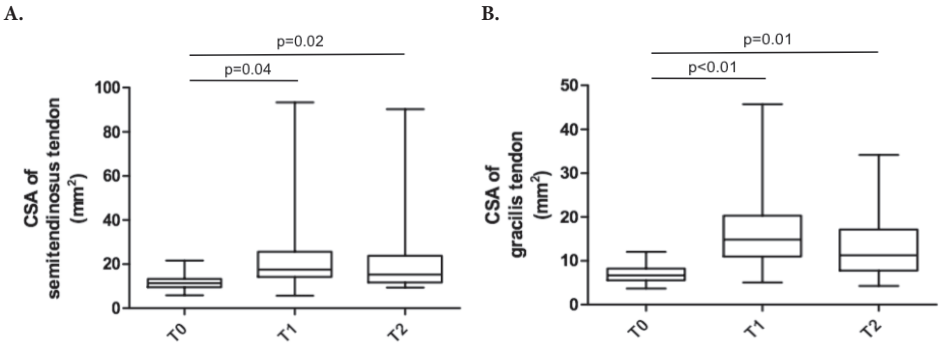

Figure 3. Remodeling of cross-sectional areas.

Boxplots displaying the absolute values of CSA (in mm²) at different time points of the (A) semitendinosus and (B) gracilis tendon.

Mean with second and third quartile. Wickets representing the lowest and highest value.

[a] T_0, baseline; T_1, 1-year follow-up; T_2, 2-year follow-up; ns, not significant.

Inter- and intraclass correlation coefficients

The two observers agreed on presence of complete regeneration and absence of regeneration in every case. Assessment of incomplete regeneration was concordant in 95% of the cases.

The interclass correlation coefficient of CSA at 1-year follow-up in the regenerated ST tendons ranged from 0.97 to 0.99 and for regenerated G tendons from 0.92 to 0.96.

The intraclass correlation coefficient of CSA at 2-year follow-up in the regenerated ST tendons ranged from 0.97 to 0.99 and for regenerated G tendons from 0.93 to 0.97.

DISCUSSION

Due to the prospective follow-up, we were able to increase knowledge about how hamstring tendons remodel. At a 2-year follow-up period ST tendons regenerated in 65.8% and

G tendons regenerated in 82.9%. Interestingly, we reported that the initial regeneration status alters in 10.5% of the patients. Additionally, we reported that regenerated hamstring tendons are hypertrophic and longer compared to their native ones.

With regeneration rates of 65.8% for the ST and 82.9% for the G tendons at a 2-year follow-up period, our findings are in line with previous work[15]. Compared to the ST tendon, the G tendon was more capable of at least partial regeneration. Surprisingly, 5 tendons that showed signs of regeneration one year after surgery, did not have signs of regeneration at the 2-year follow-up time point. We hypothesize that disappearance of the initial visualized structure at 1-year follow-up may be caused by the rupturing of the regenerated structure[10]. Another explanation for this phenomenon is that the human body may have a certain "time point of no success" and that after this time point, the body suspends its own regenerating efforts in the case of non-functionality leading to removal of the newly formed, but dysfunctional tissue. On the other hand, five patients with initially incomplete regenerated tendons, showed complete regeneration at 2-year follow-up.

Over time, various theories have been postulated aiming to explain the capacity and direction of hamstring tendons to regenerate after harvest. Analogous to repair of nerve lesions along an intact neural sheath, several authors considered the anatomic space between the fascial planes of the medial thigh as pathway for regenerating tendons[4, 12, 19]. Based on this, it has been previously hypothesized that tendons regenerate in a proximal to distal fashion along the fascial plane[7]. With presence of the partially regenerated tendons on the distal muscle ends, this study might indirectly support this hypothesis. As more fascial layers cover the ST tendon, regeneration rates of ST tendons could be expected to be higher than those of the G tendon[17]. However, in the current study we found that regeneration rates of the G are increased compared to regeneration rates of the ST. These findings are in line with previous studies[13, 20]. Furthermore, the anatomic space between medial layer I and II is not tubular in shape[2]. Taken this into account, we conclude that this pathway cannot result in a similar shape of the regenerated tendon compared to the native tendons.

A second theory is that after harvest some peritendinous tissue and tendon sheath is left at the most distal end of the ST and G tendon[5, 11]. Fibroblast precursor cells in this tissue then migrate towards the haematoma that is formed in the void space after harvest. The precursor cell then start to proliferate and start to produce collagen. In this hypothesis, the haematoma acts as a scaffold for tendon regeneration[5, 11].

In the current study, we also investigated the CSAs and musculotendinous junction shift in regenerated tendons and these findings were compared with native tendons. We found significantly increased CSAs of the regenerated tendons at both post-operative time points compared to the native tendons. This is in contradiction with Choi et al. who reported no statistical significant difference in CSA in regenerated hamstring tendons

compared with the native tendons[3]. However, the average follow-up period of their study was 3 years. This fits with the observed trend in the current study that showed an initial increase in CSA of the regenerated tendons and gradual decrease over time. Another important finding is that the range of CSAs of the regenerated tendons is increased compared to the range of the native tendons. The phenomenon of tendon hypertrophy after tendon lesion has been described before[1]. The range of increased CSAs is wide, as some regenerated tendons are hypertrophic, whereas others hardly are. This suggests that the extent of hypertrophy depends on patient characteristics. Our finding that the musculotendinous junction of both tendons shifted proximally is in line with previous studies[3, 9]. This finding is of clinical relevance as it has been previously suggested that the extent of muscle retraction might correlate with symptoms, such as cramping, weakness and pain of the posterior thigh[6-8]. Although smaller retractions might be relatively common in asymptomatic patients, patients with higher retractions might report the previous mentioned symptoms. In addition, this study reports that both hamstring tendons can regenerate independently from each other. The extent of retraction might be less if one of both tendons regenerates. Also, the fact that all kind of variations in regeneration are possible might affect clinical outcome.

The primary strength of our study is the large number of included patients. This is, to our knowledge, the first study that has investigated hamstring tendon regeneration in a prospective MRI study in 76 patients. An additional strength of our study is that hamstring tendon regeneration could be assessed at 1- and 2-year follow-up, which has never been performed before. Besides, this is the first study that did not describe regeneration as a dichotomous process, but differentiated between complete, incomplete and no regeneration.

Our study has some limitations. Some patients already underwent an MRI scan before assessment of eligibility for the study, resulting in the use of different MRI scanners. Secondly, a relatively low number of patients could be included for the analysis of the CSA and the musculotendinous junction shift. Although one might suggest that the multicenter aspect of the study and subtle surgical differences affect regeneration rates, this argument has been invalidated by our previous study[16].

Although 65.8% of the ST tendons and 82.9% of the G tendons regenerate, it remains unclear why tendons in some patients only regenerate partially or do not regenerate at all. Therefore, future studies should focus on identifying determinants and molecular mechanisms underlying regeneration processes. Furthermore, the current literature is unclear about the clinical consequences of absence of regeneration[15] and therefore possible symptoms reported by patients without regeneration should be investigated.

In conclusion, the results of this prospective multicenter MR imaging study indicate that the ST tendons regenerate in 65.8% and the G tendons in 82.9% of the patients. There was a change in extent of regeneration in 10.5% of the patients over times, in which

both improvement and deterioration were seen. Additionally, regenerated tendons are hypertrophic and longer compared to their native ones. Future research should focus understanding the cellular and molecular mechanisms of the tendon regeneration and remodeling process.

Perspective

This study provides insight in the dynamics of tendon regeneration processes, in terms of regeneration rates, morphological characteristics and possible molecular pathways of regeneration. An important finding is that an initial regenerated structure may fail over time[10]. Although regenerated tendons might be used for re-reconstruction purposes, the failure of regenerated tendons questions its quality and therefore the use of regenerated hamstring tendons in re-reconstruction procedures[21]. Another interesting observation is that regenerated hamstrings tendons are hypertrophic compared to native tendons, as has been described before for the patellar tendon[1]. The reason for this remains unclear. However, one may hypothesize that this is a protection mechanism of the human body regarding previous injuries. On the other hand, it may be postulated that the quality of the regenerated tendons may be inferior to native tendons and one needs the hypertrophic tendons to resist similar strengths as before. Also, this study contributes to the direction of future translational research in the field of tendon repair processes. As regeneration starts proximally in any case, fibroblast precursor cells in the muscles may migrate towards the hematoma that is formed in the void space after harvest[5, 11].

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CHAPTER 4

Predictive factors of hamstring tendon regeneration and functional recovery after harvesting: a prospective follow-up study

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ABSTRACT

Background: Semitendinosus and gracilis tendons may regenerate after harvesting for ligament reconstruction procedures. However, predictive factors of tendon regeneration and the extent of functional recovery remain unclear.

Purpose: To identify predictive factors for hamstring tendon regeneration and to examine the morbidity of nonregenerated hamstring tendons.

Study design: Cohort study; Level of evidence, 3.

Methods: Of the 154 patients who were included in a prospective follow-up study, 79 underwent reconstruction of the anterior cruciate ligament entailing the hamstring tendons and met the following inclusion criteria: (1) anterior cruciate ligament rupture diagnosed by physical examination and magnetic resonance imaging (MRI), (2) MRI within 6 months after trauma, (3) age between 18 and 45 years, and (4) 2-year follow-up MRI data available. Hamstring tendon regeneration was assessed as complete if a tendon-like structure could be visualized at level of joint line or more cranially. Patient characteristics - such as age, gender, body mass index, alcohol/nicotine use, activity level (Tegner scores) and functional instability (1-legged hop test) – were evaluated preoperatively and at 2 years to determine predictive factors for tendon regeneration or examine functional recovery of hamstring tendon regeneration.

Results: At 2 years' follow-up, 67.1% of the patients showed regeneration of semitendinosus tendons, 81.0% of gracilis tendons and 59.5% of both tendons. The likelihood of semitendinosus regeneration significantly decreased with aging (odds ratio [OR], 0.92 change per year of age; 95% CI, 0.84-0.99; p=0.03) and smoking (OR, 0.20; 95% CI, 0.05-0.77; p=0.02). No predictive factor was found for gracilis tendon regeneration. Regeneration of the semitendinosus and gracilis tendons was negatively related with smoking (OR, 0.22; 95% CI, 0.06-0.79; p=0.02). Patients without regeneration showed similar postoperative visual analog scale scores during physical activity, similar Tegner scores, and a significant decrease of the upper leg circumference, as compared with their preoperative results. Regardless the regeneration status, 1-legged hop test results significantly increased at 2-year follow-up.

Conclusions: Hamstring tendon regeneration occurs less frequently in older patients and in smokers. However, absence of regenerated tendons does not seem to cause a loss of function.

Key Terms: hamstring tendon regeneration; predictive factors; functional outcome; recovery; anterior cruciate ligament reconstruction.

INTRODUCTION

Anterior cruciate ligament (ACL) rupture is a common sports-related injury of the knee. Estimations of annual incidences reach up to approximately 5 to 8 per 10,000 persons[26, 31]. Numerous graft choices exist for ACL reconstruction, such as hamstring tendons autografts and bone-patellar tendon-bone (BPTB) autografts. Because of donor site morbidity and patellar tendon ruptures with the use of BPTB autografts, hamstring tendon autografts are a commonly employed option[1, 15, 22, 42].

Cross et al. were the first to describe the potential of hamstring tendons to regenerate after harvesting procedures for ACL reconstructions[12]. In a previous study, semitendinosus and gracilis tendons regenerated in at least 70% of the patients after harvesting[37]. Currently, it is unknown why some tendons lack the capacity to regenerate[37]. Mechanical load and controlled mobilization are related to a beneficial effect on tendon recovery after injury[4, 41, 44]. On the contrary, smoking[23], aging[29, 33], and alcohol use[16] are related with tendon healing failure. The role of non-steroid anti-inflammatory drugs (NSAIDs) in healing processes remains unclear[14, 32]. However, the available literature does not describe predictive factors specifically for hamstring tendon regeneration, which could be considered an altogether different process from tendon healing.

A systematic review reported on the morbidity and function loss of nonregenerated hamstrings[37]. The exact mechanism of the absence of hamstring tendon regeneration is presently unclear. Several cases were described in which patients experienced a persistent sharp pain in the dorsal aspect of the thigh in the early stage after surgery, perhaps caused by rupturing of the regenerated structure[28]. Another explanation might be that the human body suspends its regenerating efforts in case of nonfunctional tissue, resulting in a removal of the newly formed but dysfunctional tissue. Although different studies investigated the clinical response to hamstring tendon regeneration, its consequences remain unclear because of conflicting evidence. A systematic review summarized studies that examined the effect of tendon regeneration on hamstring strength and function[37], reporting conflicting evidence regarding the relationship between regeneration status and deep knee flexion. Some studies cited a deep knee flexion deficit among patients without regeneration[11, 30], whereas other studies contradicted this finding[13, 19, 27]. In addition, there is no consensus about the clinical relevance of the number of regenerated hamstring tendons. Some studies suggested that the extent of deep knee flexion deficits is limited if both tendons regenerate[11]. Other studies did not find a relationship between the number of regenerated tendons and strength deficits[19].

Nevertheless, insight into determinants of hamstring tendon regeneration and its clinical consequences is relevant for several reasons. First of all, patients voice concerns about harvesting the tendons of functional muscles and the possible accompanying functional deficits. If predicting factors are identified, the chances of hamstring tendon regeneration

could be estimated more accurately. This may affect the choice of hamstring tendons as an autograft and provide insight in the clinical consequences of regenerated hamstring tendons. After all, knowledge of determinants for hamstring tendon regeneration may lead to life style modification before surgery and changes in rehabilitation programs after surgery.
The aim of the current study was to (1) identify predictive factors for hamstring tendon regeneration and (2) examine the effect of tendon regeneration on hamstring strength and function.

METHODS

Study Population

Between January 2009 and November 2010, patients were included in the Knee Osteoarthritis Anterior Cruciate Ligament Lesion (KNALL) study: a prospective multicenter cohort study with 2 years of follow-up. Patients were recruited from 3 hospitals in The Netherlands: Erasmus MC-University Medical Center Rotterdam, Medical Center Haaglanden (The Hague) and Reinier de Graaf Gasthuis (Delft). Inclusion criteria for the KNALL study were (1) ACL rupture diagnosed by physical examination and magnetic resonance imaging (MRI), (2) MRI made within 6 months after trauma, and (3) age between 18 and 45 years. Patients who did not speak Dutch, those with previous ACL injury or intra-articular knee trauma or surgery, those with disabling comorbidity and those with already osteoarthritic changes on radiographs (Kellgren and Lawrence grade > 0) at baseline were excluded. Patients were treated operatively or nonoperatively independent of the study, according to the decision of the treating physician in accordance with the Dutch ACL guideline[24]. In the current study, operatively treated patients were included when 2-year follow-up MRI, completed questionnaires, and data of physical examination at baseline and 2-year follow-up were available. Patients were excluded if the initial treatment was other than an ACL reconstruction entailing the hamstring tendons. Written informed consent was obtained from all included patients, and the institutions' medical ethics committees approved the study.

Measurements

Two-year follow-up MRI scans were acquired on a 1.5-T MRI scanner. The patient's legs were set in a neutral position through a dedicated knee coil. Details of MRI parameters are shown in Table 1.
Hamstring tendon regeneration was evaluated by an intensively trained researcher (M.S.) who was blinded for clinical information. Hamstring tendon regeneration was assessed at 2 years' follow-up for patients who underwent surgical ACL reconstruction with the hamstring tendons. Equivocal cases were discussed with a musculoskeletal

radiologist (E.O.) and a sports medicine-trained orthopaedic surgeon (D.M.), both with more than ten years of experience, and solved with consensus. Hamstring tendon regeneration was assessed at 2-year follow-up for patients who underwent surgical ACL reconstruction with the hamstring tendons. If regenerated tendons could be visualized at the level of the joint line or more cranially, regeneration was assessed as complete. If no neotendons could be visualized on any MRI scan on any level, this was considered no regeneration. Therefore, 4 subgroups of regeneration were distinguished: regeneration of the semitendinosus tendon and gracilis tendon, regeneration of only the semitendinosus tendon, or regeneration of only the gracilis tendon, and no regeneration of either tendon.

Table 1. Parameters of magnetic resonance imaging[a].

Pulse Sequence	Slice thickness, mm	TR/TE, ms
Sagittal and coronal proton density TSE sequence	3	2700/27
Coronal T2-weighted TSE sequence with fat saturation	3	5030/71
Axial proton density and T2-weighted TSE sequence	3	3500/25/74
Sagittal 3D water excitation double-echo steady state	1.5	21.35/7.97

[a]*TE, echo time; TR, repetition time; TSE, Turbo Spin Echo.*

Harvesting procedure

After an oblique skin incision just medial to the tibial tuberosity, the subcutaneous tissue was dissected to expose the sartorius fascia. A reversed L-shaped incision on this fascia was made to free the whole pes anserinus. The gracilis and semitendinosus tendons were divided from the conjoined tendon of the pes anserinus and whip stitched. Both tendons were harvested with a closed tendon stripper. The sartorius fascia was then sutured in its anatomic position. No drains were used.

Rehabilitation

Rehabilitation consisted of full weightbearing and use protective crutches use for 6 weeks. No immobilisation or brace was applied. Return to play was considered appropriate in concurrence with the advice of the physiotherapist, on average at 8-9 months after surgery. No specific functional or quantitative protocol, such as isokinetic testing, was obligatory.

Data collection

All included patients were requested to complete several questionnaires. One trained medical doctor (B.M.) who was blinded for the regeneration status, performed a standardized physical examination and history taking at baseline and 2 years' follow-up. To evaluate determinants for hamstring tendon regeneration and the clinical consequence of nonregenerated tendons, the following factors and outcome measurements were documented:

- Patient characteristics: sex, age and body mass index at baseline. The role of the patient's sex in the process of tendon regrowth remains unclear[37]. Aging seems to affect tendon regeneration negatively[29, 33]. No data about the correlation of body mass index and tendon regeneration were available. Therefore, patient's body mass index was determined and categorized into 1 or 3 groups: <25, 25-30, and > 30 kg/m^2.
- Mechanical load: mechanical load is associated with a beneficial effect on hamstring tendon regeneration[4, 41, 44]. Therefore, preinjury and 2-year follow-up Tegner scores were analyzed as a reflection of mechanical load.
- Hospital: some studies suggested an effect of surgical proceedings; therefore, the surgeon may be a factor that affects regeneration capacity[37].
- Toxins: smoking[23] and alcohol use[16] seem to negatively affect regeneration changes. The effect of NSAIDs on regeneration remains unclear[14, 32].
- Vascular status: diabetes mellitus (DM) complicates wound healing and has negative effects on tendon-healing processes in animal studies[3, 10]. Moreover, adequate blood supply has been shown to be an important factor for ligament healing[5].
- Clinical consequences
    - o All patients completed the following questionnaires regarding pain, sports activity and knee function: visual analogue scale for knee pain (rest and physical activity)[20], Tegner scale (pretrauma level)[20], Lysholm[6, 7, 21], and International Knee Documentation Committee (IKDC) questionnaire[18, 34, 40].
    - o One-legged hop test (OLHT) was performed, and the upper leg circumference of the affected knee was determined.

Statistical analysis

All statistical analyses were performed with SPSS Statistics for Windows (v 21.0; IBM Corp). Descriptive statistics were used to describe baseline characteristics. Selection of variables was based on the available literature. To analyze predictive factors for hamstring tendon regeneration, the study population was subdivided into 3 groups based on the regeneration status. Multivariable binomial logistic regressions were used to calculate odds ratios (ORs) and 95% CIs for determinants of regeneration of hamstring tendon. Qualitative variables were coded in the following way: sex (man, 0; woman, 1), smoking (no, 0; yes, 1), alcohol use (no, 0; yes, 1), NSAID use (no, 0; yes, 1). Positive predictive values were calculated for the determinants that had a significant relationship in the multivariable model for hamstring tendon regeneration. Factors were tested for multicollinearity. To determine clinical recovery, outcomes of 4 questionnaires and physical examination were compared among the 4 regeneration subgroups (both tendons, semitendinosus tendon only, gracilis tendon only, none) and the nonoperatively treated group (control). Patients who were treated non-operatively were used as controls to examine clinical performance of native tendons after a ruptured ACL. Differences

between baseline and 2-year follow-up scores were statistically tested with paired t-tests. Significance was tested for p-value<0.05. To determine the interobserver variability, 20 randomly chosen scans were reassessed by a blinded second observer (E.O.), and an intraclass correlation coefficient (ICC; 2-way random effects model, absolute agreement) was calculated.

RESULTS

Study population

Of the 143 patients for whom MRI at 2-year follow-up was available, the baseline characteristics are presented in Table 2.

Table 2. Patient characteristics at baseline[a].

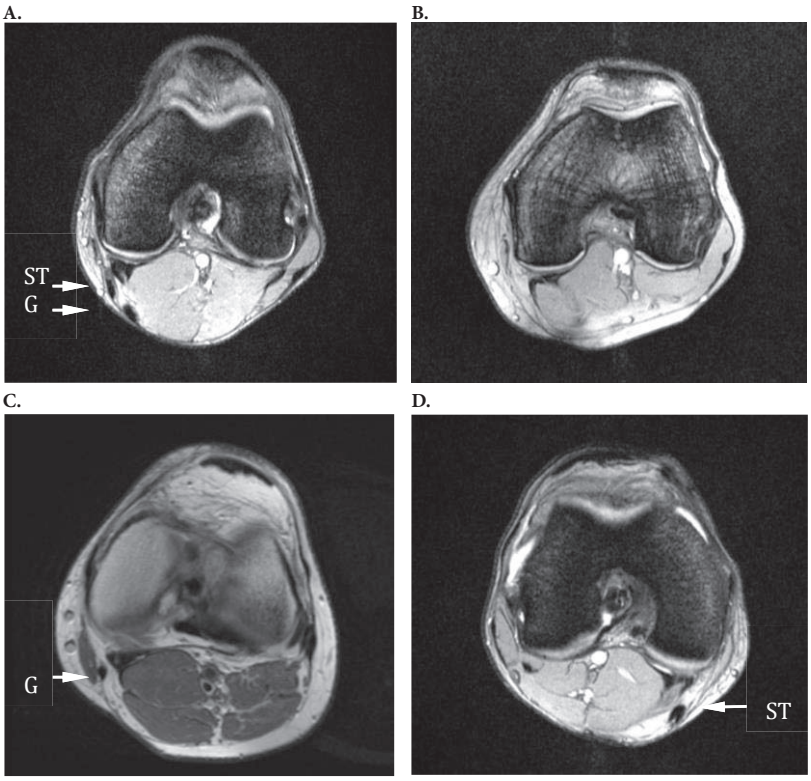
	Median (IQR) or No. (%)
Age, y	25.2 (21.4 – 32.6)
Male	94 (65.7)
Body mass index, kg/m^2	23.9 (22.0 – 26.2)
Injured side: right	76 (53.1)
Pretrauma Tegner score	9 (7 – 9)
Upper leg circumference of index knee, cm	46.7 (43.0 - 48.0)
One leg hop test of index leg, cm	55.0 (25.0 – 85.0)

[a]*IQR, interquartile range.*

During the 2-year follow-up period, 93 patients underwent an ACL reconstruction procedure. A surgical procedure entailing hamstring-tendon grafts was performed in 87 patients (93.5%), BPTB in 4 patients (4.3%), and a combination of hamstring tendon and allograft in 2 patients (2.2%). Postoperative MRI was available for 79 patients who underwent an ACL reconstruction with hamstring tendons. At 2 years' follow-up, semitendinosus and gracilis tendons regenerated in 53 (67.1%) and 64 (81.0%) patients, respectively. No tendon regeneration was reported for 9 (11.4%) patients. Figure 1 displays an overview of the regeneration subgroups. Figure 2 provides a flow chart of inclusion for eligible patients.

Predictive factors

Predictive factors were examined in cases of regeneration of the semitendinosus tendon (n=53), gracilis tendon (n=64), and both tendons (n=47). Regeneration of the semitendinosus tendon was significantly related with age (OR, 0.92 per change per year; 95% CI, 0.84-0.99; p=0.03) and smoking status (OR, 0.20; 95% CI, 0.05-0.77; p=0.02).



ST, semitendinosus; G, gracilis.

Figure 1: Representative magnetic resonance images at joint-line level after hamstrings harvesting.
***A** Left knee with regeneration of the semitendinosus and gracilis tendon.*
***B** Left knee without regeneration of the semitendinosus and gracilis tendon.*
***C** Left knee with only regeneration of the gracilis tendon.*
***D** Right knee with only regeneration of the semitendinosus tendon.*

Isolated gracilis tendon regeneration was not related with any of the analyzed predictive factors. Regeneration of both tendons was negatively related with patient's smoking status (OR, 0.22; 95% CI, 0.06-0.79; p=0.02). Table 3 represents an overview of the ORs. Because only 2 patients had diabetes mellitus and no patients were known to have abnormal cardiovascular status, we did not analyze those determinants for hamstring tendon regeneration outcome. Coefficients of determination varied from 26% (semitendinosus tendons) to 31% (semitendinosus and gracilis tendons). No multicollinearity was detected.

For the significant determinants after multivariable analyses, see Table 4, which presents the positive predictive values for tendon regeneration.

Based on the multivariate binomial logistic regression analysis, an approximation of regeneration can be assessed for the semitendinosus tendon and both tendons. For the chance of semitendinosus tendon regeneration,

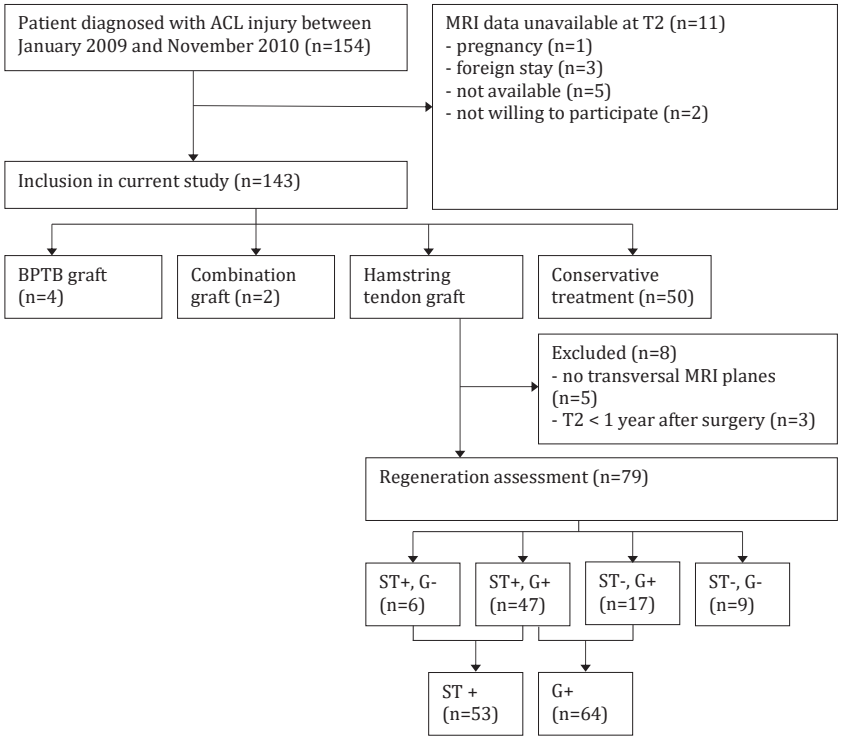


Figure 2. Flowchart.
ACL, anterior cruciate ligament; BPTB, bone-patellar tendon-bone; G, gracilis; MRI, magnetic resonance imaging; ST, semitendinosus. +, regeneration; -, no regeneration.

$$P\,(regeneration\ semitendinosus) = \frac{e^{2.245-(0.094\times age)+1.4(smoking)}}{1+e^{2.245-(0.094\times age)+1.4(smoking)}}.$$

For the chance of regenerating both tendons,

$$P\,(regeneration\ semitendinosus\ and\ gracilis) = \frac{e^{-0.619+1.363(smoking)}}{1+e^{-0.619+1.363(smoking)}}.$$

In both formulas, *e* represents the Euler number. If a patients smokes, the number 1 should be filled in the formula, whereas if the patients does not smoke, the number 0 should be filled in.

Clinical consequences

To analyze the clinical consequences of tendon regeneration, the study population was divided into 4 groups based on regeneration and 1 nonoperative group as control: semitendinosus tendon (n=53), gracilis tendon (n=64), both tendons (n=47), neither tendon (n=9), and control (n=50). When compared with preoperative scores, visual analog scale scores at physical activity significantly decreased for all groups at two years' follow-up (all p-values <0.001), except for the patients who showed no regeneration of

either tendon (p=0.14). Before trauma, Tegner scores were significantly higher for all groups versus 2-year follow-up, except for the patients with no regeneration of either tendon. Furthermore, the circumference of the upper leg decreased significantly from 47.1 cm to 45.5 cm (difference, 1.6; 95% CI of difference, 0.46-2.8; p=0.01) for patients with no regeneration of the semitendinosus and gracilis tendons, whereas patients with regeneration of at least one tendon did not show a similar decrease. One-legged hop test, Lysholm, and International Knee Documentation Committee scores significantly increased over time for all groups as compared with their preoperative scores. Table 5 presents an overview of the functional consequences and hamstring tendon regeneration.

Table 3. Multivariable analysis of possible predictive factors for hamstring tendon regeneration (n=79)[a].

	ST+ (n=53)		G+ (n=64)		ST+/G+ (n=47)	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Sex	1.7 (0.54 – 5.6)	0.35	1.4 (0.38 – 5.4)	0.60	0.69 (0.23 – 2.0)	0.50
Age	**0.92** (0.84 - 0.99)	**0.03**[b]	1.0 (0.92 – 1.1)	0.92	0.95 (0.88 – 1.0)	0.16
NSAID use	0.57 (0.15 – 2.2)	0.42	1.1 (0.23 – 5.1)	0.93	0.52 (0.15 – 1.9)	0.31
BMI[c], kg/m^2						
25-30	1.6 (0.43 – 6.3)	0.47	2.1 (0.46 – 9.3)	0.35	1.8 (0.52 – 6.1)	0.36
>30	0.27 (0.02 – 3.8)	0.33	1.0 (0.06 – 17)	0.99	0.27 (0.02 – 3.6)	0.32
Smoking	**0.20** (0.05 – 0.77)	**0.02**[b]	0.40 (0.10 – 1.6)	0.19	**0.22** (0.06 – 0.79)	**0.02**[b]
Alcohol	1.3 (0.36 – 4.9)	0.67	0.86 (0.21 – 3.5)	0.83	1.7 (0.50 – 5.7)	0.41
Surgeon[d]						
2	1.1 (0.17 – 7.1)	0.91	3.0 (0.31 – 30)	0.34	2.4 (0.41 – 14)	0.33
3	0.67 (0.08 – 5.4)	0.71	2.6 (0.21 – 33)	0.45	1.6 (0.22 – 11)	0.65
4	0.21 (0.01 – 6.2)	0.37	N/A		0.60 (0.02 – 15)	0.75
5	0.54 (0.11 – 2.6)	0.44	1.3 (0.24 – 6.7)	0.79	1.3 (0.31 – 5.5)	0.71
6	1.7 (0.20 – 15)	0.62	N/A		3.5 (0.45 – 27)	0.23

[a]*BMI, body mass index; G, gracilis; N/A, not available; NSAID, nonsteroid anti-inflammatory drug; OR, odds ratio; ST, semitendinosus; +, regeneration.*
[b]*p-value<0.05.*
[c]*Reference: <25 kg/m^2.*
[d]*Reference: surgeon 1.*

Table 4 Positive predictive values of tendon regeneration[a].

	ST+			ST+/G+		
Smoking	Yes[b]	No	PPV	Yes[c]	No	PPV
Yes	9	11	0.45	7	13	0.35
No	44	15		40	19	

[a]*G, gracilis; PPV, positive predictive value; ST, semitendinosus.; +, regeneration.*
[b]*Prior chance: 67.1% (53 of 79).*
[c]*Prior chance: 59.5% (47 of 79).*

Table 5. Functional consequences and hamstring tendon regeneration[a].

		Mean (SD)		Difference	
		T0	T2	(95% CI)	p-value
VAS (at rest)[b]	ST+ (n=53)	1.1 (1.6)	0.47 (0.9)	0.65 (0.20 – 1.1)	0.005[c]
	G+ (n=64)	1.2 (1.8)	0.52 (1.0)	0.69 (0.22 - 1.2)	0.005[c]
	ST+/G+ (n=47)	1.1 (1.7)	0.52 (0.95)	0.61 (0.11 – 1.1)	0.017[c]
	ST-/G- (n=9)	0.83 (0.85)	0.34 (0.45)	0.49 (-0.26 – 1.2)	0.17
	control (n=50)	0.71 (1.2)	0.41 (0.75)	0.29 (-0.07 - 0.66)	0.11
VAS (during movement)	ST+	2.8 (2.5)	0.73 (0.99)	2.1 (1.4 - 2.8)	<0.001[c]
	G+	2.8 (2.6)	0.86 (1.2)	1.9 (1.2 – 2.6)	<0.001[c]
	ST+/G+	2.7 (2.5)	0.80 (1.0)	1.9 (1.1– 2.6)	<0.001[c]
	ST-/G-	2.5 (2.0)	1.1 (1.3)	1.3 (-0.52 - 3.2)	0.14
	control	2.3 (2.2)	1.0 (1.5)	1.3 (0.55 - 2.0)	<0.001[c]
Tegner	ST+	8.3 (1.4)[d]	7.1 (1.9)	1.2 (0.68 - 1.6)	<0.001[c]
	G+	8.3 (1.4)[d]	6.8 (1.9)	1.5 (1.0 – 1.9)	<0.001[c]
	ST+/G+	8.3 (1.4)[d]	7.1 (1.9)	1.1 (0.66 – 1.6)	<0.001[c]
	ST-/G-	7.8 (1.4)[d]	6.4 (1.5)	1.3 (-0.10 – 32.8)	0.65
	control	7.5 (1.6)[d]	5.5 (2.0)	2.0 (1.4 - 2.6)	<0.001[c]
Lysholm	ST+	77.2 (13.1)	93.0 (7.2)	15.8 (12.5 – 19.2)	<0.001[c]
	G+	75.4 (16.0)	92.8 (7.3)	17.4 (13.5 – 21.2)	<0.001[c]
	ST+/G+	76.9 (13.7)	92.8 (7.4)	15.9 (12.1 – 19.6)	<0.001[c]
	ST-/G-	64.6 (11.7)	87.8 (15.8)	23.2 (9.1 – 37.3)	0.005[c]
	control	74.6 (16.8)	91.6 (12.3)	17.0 (11.6 - 22.4)	<0.001[c]
IKDC	ST+	54.4 (14.9)	87.6 (10.4)	33.2 (28.9 – 37.5)	<0.001[c]
	G+	52.9 (16.3)	87.8 (11.2)	34.9 (30.4 – 39.3)	<0.001[c]
	ST+/G+	54.2 (14.9)	87.9 (10.3)	33.7 (29.0 – 38.4)	<0.001[c]
	ST-/G-	50.8 (11.3)	85.2 (16.0)	35.4 (18.7 – 50.0)	0.001[c]
	control	59.2 (19.0)	84.3 (14.7)	25.1 (10.2 – 30.9)	<0.001[c]
One-legged hop test (cm)	ST+	53.2 (38.7)	110.8 (29.4)	57.6 (46.8 – 68.3)	<0.001[c]
	G+	52.5 (38.9)	113.1 (30.9)	60.5 (50.7 -70.4)	<0.001[c]
	ST+/G+	54.1 (38.6)	111.2 (30.1)	57.1 (46.2 – 68.1)	<0.001[c]
	ST-/G-	37.4 (40.2)	94.6 (26.6)	57.1 (31.1 – 83.1)	0.001[c]
	control	57.9 (37.9)	101.3 (36.5)	43.3 (33.4 - 53.3)	<0.001[c]
Circumference upper leg (cm)	ST+	45.6 (4.8)	45.4 (3.7)	0.19 (-0.83 – 1.2)	0.71
	G+	47.3 (12.8)	45.7 (3.6)	1.6 (-1.5 – 4.8)	0.31
	ST+/G+	45.8 (5.0)	45.6 (3.8)	0.23 (-0.90 – 1.4)	0.68
	ST-/G-	47.1 (4.7)	45.5 (4.5)	1.6(0.46 – 2.8)	0.01[c]
	control	46.6 (5.5)	46.3 (4.4)	0.31 (-0.62 - 1.3)	0.50

[a]*G, gracilis; IKDC, International Knee Documentation Committee; ST, semitendinosus; T0, preoperative; T2, 2-year follow-up; VAS, visual analog scale; + regeneration; -, no regeneration.*

[b]*Sample sizes apply to each grouping.*

[c]*$p<0.05$.*

[d]*Pretrauma Tegner.*

Inter- and intracorrelation coefficients

The 2 observers agreed on presence of complete regeneration and absence of regeneration in every case. Assessment of incomplete regeneration was concordant in 95% of the cases. The interclass correlation coefficient of cross-secitonal areas in the regenerated semitendinosus tendons ranged from 0.97 to 0.99 and for regenerated gracilis tendons from 0.92 to 0.96.

The intraclass correlation coefficient of cross-sectional areas in regenerated semitendinosus tendons ranged from 0.97 to 0.99 and for regenerated gracilis tendons from 0.93 to 0.97.

DISCUSSION

Hamstring tendon regeneration occurs in at least 70% of the patients[37]. The results of this prospective observational follow-up study show that hamstring tendon regeneration occurs significantly less frequent in patients who smoke. Furthermore, semitendinosus tendons are less likely to regenerate in older patients. If none of the harvested tendons regenerated, patients did not report improved physical activity and a significant decrease of their upper leg circumference was observed.

In the current study, semitendinosus tendons regenerated more often than gracilis tendons. This finding is in line with previous studies[37-39]. To explain the difference in regenerative capacity, we developed the following hypothesis: that hamstring tendon regeneration occurs behind the deep layer of the thigh fascia. Regarding this fascia, the gracilis tissue plane is covered and protected to a lesser extent than to the semitendinosus tendon. This anatomic difference may explain inferior gracilis tendon regeneration rates versus those of the semitendinosus tendons.

Although previous literature described several determinants for tendon healing, potential predictive factors for hamstring tendon regeneration have not been investigated; therefore, this study is the first to evaluate potential predictive factors for hamstring tendon regeneration based on known factors for tendon healing. For regeneration of the semitendinosus tendon, we identified age and smoking as predictive factors. Age-related changes in tendons include loss of cellularity, loss of vascularity, and fatty infiltration[17]. The latter two are mainly thought to be responsible for less regenerative capacity in tendons. The exact mechanism of smoking on hamstring tendon regeneration remains unclear. It could be that nicotine, as a known major vasoconstrictor, affects tendons' regeneration chances by decreasing the blood supply to former harvest sites[25]. However, nicotine use could also be a marker for unhealthy lifestyles. Nonetheless, based on these results, it remains hard to predict an individual's capacity for hamstring tendon regeneration after harvest procedure. As with common orthopaedic conditions, we suggest a model of intrinsic and extrinsic factors that produce an indication of susceptibility for regenerating

processes. However, identifying the cause and genetic linkage of orthopaedic phenotypes has proven to be complex and requires further investigation. Therefore, the current study points out that regeneration of the semitendinosus is related to patient's age and smoking habits, but it may be that genetic factors also contribute to one's regeneration capacity.

This study is the first to examine functional consequences of hamstring tendon regeneration in 5 subgroups: regeneration of 1 tendon only, regeneration of both tendons, no regeneration of either tendon, and a nonoperatively treated group (control). Although the primary function of the hamstring muscles is to flex the knee or to decelerate its extension, the hamstring muscles control anterior translation of the tibia, sharing the stress with the ACL. However, we found that all patients experience better knee stability at 2 years' follow-up, regardless of regeneration status of the hamstring tendons. Second, several previous studies used the 1-legged hop test for distance to examine strength and confidence in the tested leg[34, 35]. In the current study, all groups showed a significant increase in the 1-legged hop test results, suggesting that the number of regenerated tendons does not affect clinical performance. An increase of 1-legged hop test results has been reported[2]; however, this study did not differentiate between patients with and without regenerated tendons. In addition, Choi et al. reported no statistically significant difference between the number of regenerated tendons and 1-legged hop test results[11]. This is in line with the findings of the current study.

Furthermore, we found that the circumference of the operated upper leg is significantly decreased among patients without regeneration versus patients showing regeneration of one or more hamstring tendons. A previous study reported that the majority of the upper leg atrophy involves the semitendinosus and gracilis muscles[36], although this could not be confirmed with measurements in the current study.

A previous study of 45 patients investigated the relationship among tendon regeneration, flexor strength, and functional tests at a minimum follow-up of 2 years, reporting that individual tendon regeneration was associated with fewer knee flexion deficits at 70° and with improved performances on the carioca test[11]. Taken together, the results may suggest that lack of regeneration results in knee flexion deficits because of muscle atrophy of the harvested tendons.

The current study confirms previous studies' findings of a significant decrease of upper leg circumference in the case of no tendon regeneration, which suggests muscle atrophy[8, 43]. These studies showed a compensatory hypertrophy of the biceps femoris. However, this could not adequately compensate the loss of muscle volume measured in the harvested medial hamstrings[9, 36]. Unfortunately, most of these studies compared clinical outcomes postoperatively regardless of an individual's regeneration status. In addition, only relatively short-term follow-up studies are available. So, despite some strong indications to the clinical relevance of hamstring tendon regeneration, it remains to be seen if different

degrees of muscle atrophy and tendon regeneration will have any clinically relevant effect on patients at longer-term follow-ups.

The strengths of the current study are its prospective design, availability of baseline and follow-up MRI, extensive physical examination, and questionnaires at baseline and follow-ups. Because of these strengths, we were able to identify predictive factors and clinical consequences of hamstring tendon regeneration for different subgroups.

This study also has some limitations. The parameters used to evaluate the clinical consequences of regeneration may be debatable, as they may be not specific for hamstring tendons. However, there is currently no test to evaluate the function of the semitendinosus and gracilis muscles. Although there are no functional consequences, determining muscle function with Biodex measurements may be useful. Another limitation of our study is that patients showing regeneration of both t tendons were included in analysis for semitendinosus and gracilis regeneration separately; therefore, those 3 groups have a certain overlap.

In conclusion, the current study reported that semitendinosus and gracilis tendons regenerated in 67.1% and 81.0% of patients, respectively. Furthermore, it points out that regeneration of the semitendinosus tendon is related with an individual's age and smoking habits. Likewise, regeneration of both hamstring tendons is negatively related to smoking habits. However, absence of regenerated tendons does not seem to cause a loss of function.

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CHAPTER 5

Functional polymorphisms within the inflammatory pathway regulate expression of extracellular matrix components in a genetic risk dependent model for anterior cruciate ligament injuries

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ABSTRACT

Objectives: To investigate the functional effect of genetic polymorphisms of the inflammatory pathway on structural extracellular matrix components (ECM) and the susceptibility to an anterior cruciate ligament (ACL) injury.

Design: Laboratory study, case-control study.

Methods: Eight healthy participants were genotyped for interleukin (IL)*1B* rs16944 C>T and *IL6* rs1800795 G>C and classified into genetic risk profile groups. Differences in type I collagen (*COL1A1*), type V collagen (*COL5A1*), biglycan (*BGN*) and decorin (*DCN*) gene expression were measured in fibroblasts either unstimulated or following IL-1β, IL-6 or tumor necrosis factor (TNF)-α treatment.

Moreover, a genetic association study was conducted in: (i) a Swedish cohort comprised of 116 asymptomatic controls (CON) and 79 ACL ruptures and (ii) a South African cohort of 100 CONs and 98 ACLs. Participants were genotyped for *COL5A1* rs12722 C>T, *IL1B* rs16944 C>T, *IL6* rs1800795 G>C and *IL6R* rs2228145 G>C.

Results: *IL1B* high-risk fibroblasts had decreased *BGN* (p=0.020) and *COL5A1* (p=0.012) levels after IL-1β stimulation and expressed less *COL5A1* (p=0.042) following TNF-α treatment. Similarly, unstimulated *IL6* high-risk fibroblasts had lower *COL5A1* (p=0.012) levels than *IL6* low-risk fibroblasts.

In the genetic association study, the *COL5A1-IL1B-IL6* T-C-G (p=0.034, Haplo-score: 2.1) and the *COL5A1-IL1B-IL6R* T-C-A (p=0.044, Haplo-score: 2.0) combinations were associated with an increased susceptibility to ACL injury in the Swedish cohort when only male participants were evaluated.

Conclusions: This study shows that polymorphisms within genes of the inflammatory pathway modulate the expression of structural and fibril-associated ECM components in a genetic risk dependent manner, contributing to an increased susceptibility to ACL injuries.

Key words: Anterior cruciate ligament injury; Extracellular Matrix; Genetics; Polymorphisms; Personalized Medicine.

INTRODUCTION

Anterior cruciate ligament (ACL) rupture is a common sports-related injury of the knee[1]. The ability of the ACL to maintain its extracellular matrix (ECM) integrity is critical to its function to effectively resist mechanical loads and prevent injury[2]. Loading activates matrix-remodeling pathways to maintain ECM homeostasis, such as the inflammatory pathways (Figure 1). Therefore, it is not surprising that polymorphisms within these pathways contribute to the susceptibility of ACL injuries[3, 4].

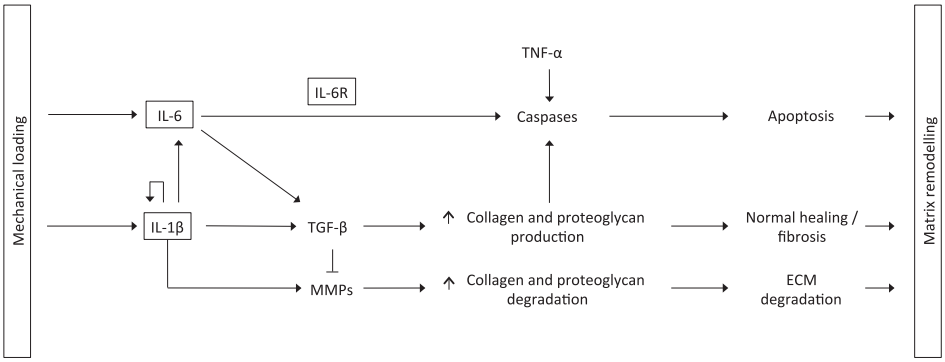

Figure 1. Schematic representation of the proposed downstream effects of cytokines IL-1β and IL-6 which are upregulated in response to mechanical loading of a ligament [3, 4, 28, 32].
Activation/upregulation is represented by a pointed arrow head (→) and inhibition/down regulation is represented by a perpendicular line at the end (---|). The boxed molecules are the ones investigated in the current study. Abbreviations: ECM, extracellular matrix; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; MMPs, matrix metalloproteinases; ROS, reactive oxygen species; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor.

Type V collagen is a functionally important collagen for the maintenance of tissue structure and integrity. The major isoforms consists of two α1 (V) and one α2 (V) chains encoded by *COL5A1* and *COL5A2* respectively[5]. Polymorphisms within the 3'UTR of *COL5A1* were previously implicated in ACL rupture[6] and tendinopathy[7]. In addition, polymorphisms within genes encoding the α1(I) chain of type I collagen (*COL1A1*)[8], biglycan (*BGN*)[9] and decorin (*DCN*)[9] were associated with ACL injury susceptibility. Together, these molecules form the basic building blocks of the ECM and are involved in collagen fibrillogenesis.

Interleukin (IL) -1β is a pro-inflammatory cytokine encoded by *IL1B* and up-regulates the production of matrix metalloproteinases, regulating the degradation of specific ECM components, such as collagen types V and X[10]. In addition, IL-1β induces its own expression and the expression of other pro-inflammatory cytokines such IL-6 (**Figure 1**)[11]. The C-allele of the *IL1B* promoter polymorphism rs16944 C>T increases IL-1β

mRNA expression levels[12] and is hypothesised to increase susceptibility to tendinopathy and ACL injury[3, 4].

IL-6 is known to induce apoptotic cell death[15] affecting the production of extracellular matrix components and thereby the ECM integrity. Polymorphisms within the *IL6* gene that increase IL-6 expression, such as the G-allele of the *IL6* rs1800795 G>C polymorphism, can therefore potentially be associated with increased risk of ligament injuries. IL-6 needs to bind and form complexes with the interleukin-6 receptor (IL-6R) in order to exert its biological function. IL-6R exists as two isoforms: a membrane-bound receptor and a soluble receptor. *IL6R* rs2228145 A>C is located in the cleavage site and is thought to affect cleavage efficiency. The A-allele is associated with decreased levels of soluble IL-6R and an increased response to IL-6[13]. Therefore, we hypothesize that the *IL6R* rs2228145 AA genotype is associated with an increased susceptibility to ligament injury.

Although currently no genetic loci within the gene encoding tumor necrosis factor α (TNF-α) have been associated with either ACL injuries or tendinopathies, this pro-inflammatory protein is considered to be key in the inflammatory pathway[14]. The biological function of TNF-α is executed after binding to its receptor, the tumor necrosis factor receptor superfamily member 1A (TNFRSF1A). Similar to IL-6, TNF-α is involved in apoptosis and thereby possibly contributes to matrix remodeling capacity[14].

The main aim of the current study was to investigate the effects of specific genetic loci within the inflammatory pathway on the production of ECM components in an injury risk model. Additionally, the association of these genetic loci with susceptibility to ACL injury was evaluated in two independent populations of different ancestry. Based on the *a priori hypothesis* it was proposed that the *IL1B* rs16944 CC and the *IL6* rs1800795 GG downregulate the production of ECM components and should therefore be associated with an increased susceptibility to ligament injuries.

METHODS

All participants completed questionnaires regarding personal details, medical history, sporting history and a family history of tendon and ligament injury. Written informed consent was obtained from all participants according to the Declaration of Helsinki. Ethics approval was attained from the Human Research Ethics Commission (HREC) of Faculty of Health Sciences, University of Cape Town, South Africa (HREC 164/2006 and 645/2014) and the Regional Ethical Review Board in Umeå, Sweden (dnr. 2011-200-31M), where relevant.

For the in vitro work eight healthy, unrelated South African participants of self-reported Caucasian ancestry with no history of musculoskeletal soft tissue injuries were recruited. Venous blood and skin biopsies were taken from each participant.

For the Swedish cohort 195 physically active and unrelated participants (age 19-65 years) were recruited between 2011 and 2013 from either the Västerbotten or Norrbotten regions of Sweden, via the orthopedic clinics in two major hospitals in the cities of Umeå: Västerbotten and Luleå: Norrbotten. The majority of the participants were recruited from a long-term follow-up of ACL injury[15]. This cohort consisted of 79 participants with ACL rupture (SWE-ACL) and 116 asymptomatic participants without any history of ACL or tendon injury (SWE-CON). ACL ruptures were diagnosed based on physical examination, magnetic resonance imaging and arthroscopically confirmed at the University hospital in Umeå. Mechanism of injury data was categorized into direct contact, indirect contact, non-contact and skiing sports as previously defined[16]. All 79 cases reported a non-contact mechanism (SWE-NON) of injury.

For the South African cohort 198 physical active and unrelated participants were recruited from South Africa as previously described[17]. This cohort comprised of 100 asymptomatic controls (SA CON) and 98 participants with an ACL rupture (SA ACL) of which 51 reported a non-contact mechanism of injury.

A previously described protocol with slight modifications[18] was used to extract genomic DNA from venous blood. Participants participating in the *in vitro* study were genotyped for the *IL1B* rs16944 C>T and *IL6* rs1800795 G>C polymorphisms. Restriction fragment length polymorphism (RFLP) analysis was used for *IL1B* rs16944 (*AvaI*)[4] while custom designed fluorescence-based Taqman PCR assays (Applied Biosystems, Foster City, CA, USA) were used to genotype *IL6* rs1800795. Several controls were included in the genotyping protocols which included repeated controls and negative controls. Two independent researchers scored genotype results obtained from RFLP analysis. If no consensus was reached, or genotyping was not possible, samples were re-analysed. TaqMan PCR determines genotype calls automatically, however were manually checked by a researcher. In general, all samples were analysed only once. Based on their genotypes, participants were either classified in the high-risk or low-risk profile group. More specifically, the *IL1B* TT and CT genotypes were considered as low-risk, whereas the CC genotype was considered as high-risk. Additionally, the *IL6* CC and GC were classified in the low-risk group, and the GG-genotype was classified in the high-risk group.

Participants for the genetic association study were additionally screened for *COL5A1* rs12722 C>T and *IL6R* rs2228145 A>C. Genotyping of all four single nucleotide polymorphisms (SNPs) was conducted in the current study on all samples in both the Swedish (n=195) and the South African cohort (n=198). It should be noted that the DNA samples of the South African cohort were previously collected[17]. Restriction fragment length polymorphism (RFLP) analysis was used for the *COL5A1* rs12722 (*BstuI)*, and

IL6R rs2228145 (*HindIII*) SNPs. All SNPs: *COL5A1* rs12722 C>T, *IL1B* rs16944 C>T, *IL6* rs1800795 G>C and *IL6R* rs2228145 A>C were selected based on their previously reported genetic associations with risk of ACL ruptures and Achilles tendinopathy[19, 20].

To establish primary fibroblast cultures, skin biopsies were processed according to a modified Baumgarten protocol[21]. Human dermal fibroblasts were cultured to 70% confluency in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA, USA) with 200 units/mL penicillin, 100 µg/mL streptomycin, 3.97 mM GlutaMAX (Gibco) and 10% FBS. Cells were serum-starved for 8h in DMEM and subsequently treated with 10ng/ml human recombinant (hr) IL-6[22], 20ng/ml hrIL-1β[23] or 10ng/ml hrTNF-α[22] (all from Peprotech, Rocky Hills, NJ, USA). After 24h, cells were washed twice in ice-cold Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, Saint Louis, MO, USA) and frozen at -80°C until ready for RNA extraction, using the RNAeasy kit (Qiagen, Venlo, The Netherlands). Subsequently, a cDNA synthesis kit including a recombinant RNAse inhibitor (Thermo Scientific) using oligo (dT)s as primers was used.

A SYBR green-based buffer (Thermo Scientific), 10ng of cDNA, and primers specific for the transcript of interest, to a final concentration of 500nM each were mixed. PCR cycles were as follows: (2'30" at 50°C: 2'30" at 95°C) x1, (15" at 95°C: 30" at 60°C) x50 followed by melt-curve analysis (95°C-60°C-95°C). RT-PCR analyses were performed using a Quantstudio3 real-time PCR machine (Thermo Scientific). The mRNA expression levels of structural matrix components, such as *COL5A1, COL1A1, DCN* and *BGN* were assessed for each sample including components of the inflammatory pathway, namely *IL1B, IL6R1, IL6, IL6R* and *TNFRSF1A* (Invitrogen, Carlsbad, CA, USA) (Supplementary Table 1). Cofilin (Invitrogen) was previously found stable and linearly correlated with RNA quantity (data not shown), and was therefore used to normalize qPCR data. Both positive and negative controls were always included.

Statistical analyses were performed with the programming environment R (R Development Core Team). In the cytokine stimulation experiments, statistics were performed using Unpaired, two-tailed Student's t-test. Power analysis was performed using QUANTO v.1.2.4 (http://biostats.usc.edu/software) to calculate sample size for the Swedish cohort. Assuming minor allele frequencies between 0.1 and 0.5 a sample size of 79 cases would be adequate to detect an allelic odds ratio (OR) of 2.3 and greater at a power of 80%. Basic descriptive statistics were compared using the one-way analysis of variance to detect significant differences between characteristics of the SWE-CON group and the SWE-NON group. The R package *genetics*[24] and *SNPassoc*[25] were used to analyse differences in genotype and allele frequencies between the groups and to calculate Hardy-Weinberg equilibrium probabilities. Inferred allele constructs were created for *COL5A1-IL1B-IL6-IL6R* genes from both the Swedish and South African genotype data respectively using the *haplo.stats* package in R[26]. The analysed models were based on previously reported associations[3, 4].

RESULTS

Fibroblasts derived from 8 donors had a high-risk genetic profile for either *IL1B* rs16944 C>T or *IL6* rs1800795 G>C (Supplementary Table 2). No significant differences in basal expression were observed in any of the ECM genes when fibroblasts were classified based on *IL1B* genotypes (Figure 2A). A reduced (p=0.012) *COL5A1* expression was noted in *IL6* high-risk fibroblasts compared to low-risk fibroblasts. As for the cytokine-related genes, we found that *TNFRSF1A* was less (p=0.003) expressed in the untreated *IL6* high-risk fibroblasts (Supplementary Figure 1A, B).

In *IL1B* high-risk fibroblasts, *COL5A1* (p=0.012) and *BGN* (p=0.020) expression were reduced following hrIL-1β. Additionally, treatment with hrTNF-α resulted in decreased *COL5A1* (p=0.042) levels (Figure 2G). In untreated *IL6* high-risk fibroblasts, *COL5A1* was reduced (p=0.012) compared to *IL6* low-risk fibroblasts (Figure 2B). No stimulation of the fibroblasts with hrIL-6 (Supplementary Figure 1C, D), hrIL-1β(Supplementary Figure 1E, F) or hrTNF-α (Supplementary Figure 1G, H) did not significantly alter the expression of any of the cytokine-related genes analysed.

Polymorphisms within *IL1B* and *IL6* alter the expression of structural and fibril-associated ECM components and herewith possibly modulate the susceptibility of ligament injuries. Therefore, these associations were further investigated in other population groups from (i) Sweden and (ii) an indigenous mixed ancestry population from South Africa.

The South African population was previously described in detail[17]. Swedish participants were matched for height, body mass and body mass index (BMI) (Supplementary Table 3). However, participants in the SWE-CON group consisted of significantly less men (34.5%, n=40) than the SWE-NON group (54.4%, n=43, p=0.014) and were significantly older (44.7 ± 11.9, n=114) than participants in the SWE-NON group (36.5 ± 13.7, n=78, p<0.001). Differences in medical and family history are displayed in Supplementary Table 4. No significant genotype effects were noted on age, sex, height, body mass or body mass index for the investigated polymorphisms (Supplementary Table 5).

No significant differences in genotype or allele frequency distributions were observed for either *COL5A1* rs12722 C>T, *IL1B* rs16944 C>T and *IL6* rs1800795 G>C in both the South African and Swedish cohorts (Table 1). However, for the South African cohort the *IL6R* rs2228145 A>C CC genotype was significantly overrepresented (p=0.028) in the SA-CON group (13%, n=12) compared to the SA-ACL group (3%, n=3). Although not significant (p=0.054), a similar trend was observed when comparing the SA-CON group (13%, n=12) to the SA-NON subgroup (11%, n=6). Furthermore, the genotype and allele frequency distributions significantly differed between the South African and Swedish cohorts (Supplementary Table 6) for all the polymorphisms tested. Therefore, cohorts could not be combined for further analysis.

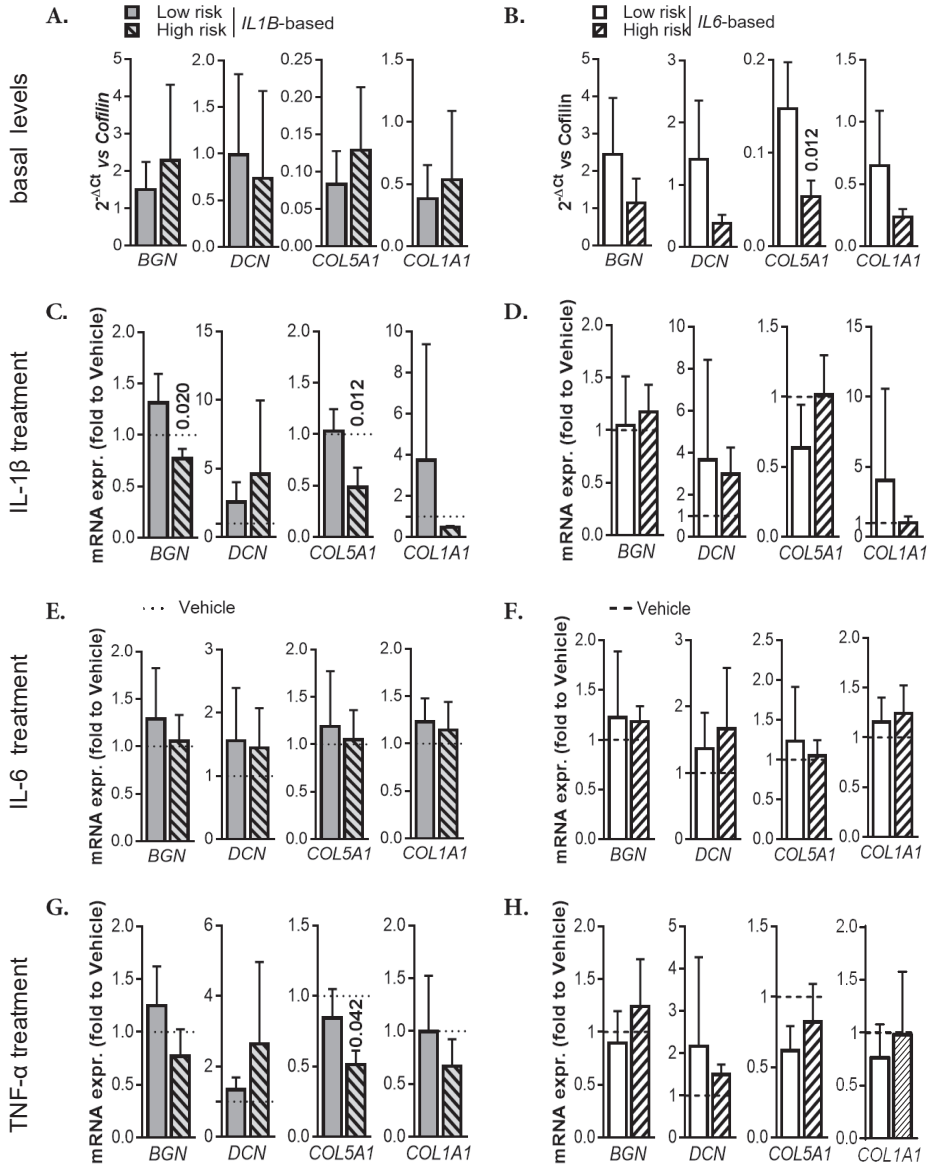

Figure 2: mRNA expression of extracellular matrix genes in stimulated and unstimulated fibroblasts.
*Primary human fibroblasts were obtained from 8 healthy volunteers and classified in high-risk or low-risk for ligament injuries based on IL1B rs12722 C>T (**A, C, E, G**) and IL6 rs1800795 G>C (**B, D, F, H**). Fibroblasts were treated with vehicle (PBS) to evaluate basal levels (**A, B**)or hr-IL-6 (**C, D**), hrIL-1β (**E, F**) or hrTNF-α (**G, H**) to evaluate fold-response to the treatment, compared to vehicle of the expression of extracellular matrix genes type I collagen α1 (COL1A1), type V collagen α1 (COL5A1), decorin (DCN), biglycan (BGN). Data is presented as (**A, B**) $2^{-\Delta Ct}$ to assess gene expression compared to CFL1 (housekeeping gene) or (**C-H**) fold to vehicle (dotted lines). Data is presented as mean with standard deviation (SD). Unpaired two-tailed Student's t-test. P-values in bold typeset indicate significance (p< 0.050).*

Table 1. Genotype and minor allele frequency distributions, and p-values for Hardy-Weinberg exact test for the four selected polymorphisms in the control (SWE-CON and SA-CON), the anterior cruciate ligament (SA-ACL) rupture group and ACL subgroup with a noncontact (SWE-NON, and SA-NON) mechanism of injury within the South African and Swedish cohorts[a].

		South Africa					Sweden		
		SA-CON	SA-ACL	p-value[a]	SA-NON	p-value[b]	SWE-CON	SWE-NON	p-value[b]
	n	96	93		48		109	77	
	CC	37 (36)	27 (25)	0.866	35 (17)	0.793	22 (24)	23 (18)	0.773
COL5A1 **rs12722 C > T**	CT	49 (47)	52 (48)		54 (26)		43 (47)	47 (36)	
	TT	14 (13)	22 (20)		10 (5)		35 (38)	30 (23)	
	T allele	38 (73)	47 (88)	0.851	38 (36)	1.000	56 (123)	53 (82)	0.617
	HWE	0.829	0.824		0.367		0.241	0.648	
	n	*98*	*93*		*48*		*112*	*78*	
	CC	24 (24)	27 (25)	0.530	27 (13)	0.923	39 (44)	44 (34)	0.799
IL1B **rs16944 C > T**	CT	47 (46)	52 (48)		44 (21)		41 (46)	40 (31)	
	TT	29 (28)	22 (20)		29 (14)		20 (22)	17 (13)	
	T allele	52 (102)	47 (88)	0.411	51 (49)	0.971	40 (90)	37 (57)	0.542
	HWE	0.549	0.836		0.395		0.120	0.224	
	n	*98*	*98*		*51*		*113*	*77*	
	GG	72 (71)	64 (63)	0.445	67 (34)	0.339	22 (25)	26 (20)	0.606
IL6 **rs1800795 G > C**	GC	26 (25)	31 (30)		27 (14)		59 (67)	52 (40)	
	CC	2 (2)	5 (5)		6 (3)		19 (21)	22 (17)	
	C allele	15 (29)	20 (40)	0.185	20 (20)	0.369	48 (109)	48 (74)	1.000
	HWE	1.000	0.539		0.373		0.060	0.821	
	n	*95*	*95*		*49*		*112*	*76*	
	AA	54 (51)	58 (55)	**0.028**	55 (27)	0.054	53 (59)	46 (35)	0.618
IL6R **rs2228145 A > C**	AC	34 (32)	39 (37)		43 (21)		37 (41)	46 (35)	
	CC	13 (12)	3 (3)		2 (1)		11 (12)	8 (6)	
	C allele	29 (56)	23 (43)	0.161	23 (23)	0.346	29 (65)	31 (47)	0.779
	HWE	0.082	0.385		0.257		0.253	0.599	

Genotype and allele frequencies are expressed as a percentage with the number of participants (n) in parentheses.
[a]CON vs. ACL (unadjusted p-value).
[b]CON vs. NON (unadjusted p-value).
P-values in bold typeset indicate significance (p< 0.050).

Allele combinations were inferred for *COL5A1-IL1B-IL6 and COL5A1-IL1B-IL6R*. For each of the two allele combinations, eight possible constructs were inferred at a frequency above 4%. For the South African cohort, no significant differences in the frequency distributions of these combinations were observed when all participants were evaluated or when only male or only female participants were compared (Supplementary Figure 2). The frequency distributions for the *COL5A1-IL1B-IL6* and the *COL5A1-IL1B-IL6R* allele combinations were similar between the control and cases when all participants or only

the female participants in the Swedish cohort were compared (Supplementary figures 3A and 3C). However, for the *COL5A1-IL1B-IL6* allele combination, when only males participants were evaluated, the T-C-G combination was significantly underrepresented (p=0.034 Haplo-score: 2.1) in the SWE-CON (7.7%, n=3) compared to the SWE-NON (18.0%, n=8) group (Supplementary Figure 3B). Furthermore, the frequency distributions for the *COL5A1-IL1B-IL6R* allele combinations, showed the T-C-A combination to be significantly underrepresented (p=0.044, Haplo-score: 2.0) in the SWE-CON (28.0%, n=11) compared to the SWE-NON (14.0%, n=6) group when only the male participants were compared in the Swedish cohort.

DISCUSSION

Considering the ligament as an integrative part of the knee joint, it is plausible that the ACL is subjected to cues derived from its surrounding anatomical structures, such as the synovium or synovial fluid. It is proposed, that as a response to repetitive mechanical overloading, macrophages might infiltrate tissues surrounding the ligaments[27]. Thereby, potentially exposing the ligamentocytes to an additional amount of specific inflammatory cytokines as part of the matrix remodeling mechanism. It is interesting, that some of the genetic susceptibility loci implicated in tendon and ligament injuries encode proteins involved in the homeostatic regulation of ECM components of both tendon and ligament, and components of the proinflammatory pathway[20]. This study, therefore used a hypothesis-based approach to evaluate the potential impact of the inflammatory pathway on modulating susceptibility to ligament injuries using an *in vitro* risk associated model, complimented with a genetic association approach.

For the functional *IL1B* rs16944 polymorphism, treatment with hrIL-β resulted in a 1.3-fold decrease (p=0.020) of *BGN* and a 2.1-fold (p=0.012) decrease of *COL5A1* in a genetic risk associated dependent manner. In addition, hrTNF-α treatment displayed a 2.0-fold (p=0.042) reduction in *COL5A1* mRNA levels in the fibroblasts with an *IL1B* rs16944 CC genotype. We suggest that, given an inflammatory micro-environment where these cytokines are abundant, matrix production is differently affected in *IL1B* high-risk compared to *IL1B* low-risk genetic profiles.

The *IL6* rs1800795 G-allele increases IL-6 mRNA expression levels, inducing apoptosis[28] which might decrease the production of ECM components. Our experiments indirectly support this hypothesis since fibroblasts having the *IL6* rs1800795 GG genotype displayed a 2.8-fold reduction (p=0.012) in *COL5A1* mRNA. Although not significant, a similar trend (p=0.07) was observed for other associated ECM components such as *DCN*. This is an important finding, since both *COL5A1* and *DCN* are required for normal fibrillogenesis[29].

At basal levels, the expression of proinflammatory genes was relatively low for all groups. However, with the exception of *TNFRSFA1* mRNA expression, mRNA levels of all the investigated cytokines were increased on average between 1.03 and 6109 fold in all the groups after treatment with hrIL-1β (Supplementary Figure 1C, D), hrIL-6 (Supplementary Figure 1 E, F) and TNF-α (Supplementary Figure 1G, H). More specifically, treatment with hrIL-1β significantly upregulated *IL1B* and *IL6* mRNA levels 3690 and 3948-fold respectively, although no statistically significant differences in their expression were noted between the high- and low-risk groups. This is in agreement with the hypothesis as shown in Figure 1 and with previous work[11].

These results support the proposal that polymorphisms within *IL1B* and *IL6* alter the expression of structural and fibril-associated ECM components and herewith possibly modulate the susceptibility to ligament injuries. This holds true in specific cohorts where these loci were implicated in risk models for the susceptibility to tendon and ligament injuries[3, 4]. These associations were therefore evaluated in two independent population groups from different ancestries, one from Sweden and the other from South Africa in an attempt to identify the susceptibility significance of these genetic loci in different populations. In the South African cohort, the *IL6R* rs2228145 CC genotype was significantly overrepresented (p=0.028) in the controls, compared to individuals that sustained an ACL injury. Although the CC genotype frequencies appeared to be similar in our Swedish cohort and in a previously reported South African Caucasian cohort[3], it did not reach the level of significance. As shown previously, the *COL5A1-IL1B-IL6* T-C-G and the *COL5A1-IL1B-IL6R* T-C-A allele combinations were found to be associated with an increased susceptibility to sustain an ACL rupture in the Swedish cohort when only male participants were evaluated[3, 4]. These associations were not reproduced in the South African cohort evaluated in this study, which might be explained by the different genetic background of the cohorts, as illustrated by the significant differences in genotype frequencies. Based on our power analysis, the sample size in this study is adequate to detect an allelic odds ratio (OR) of 2.4 at approximately 80% statistical power for Type 1 error detection. Although the study is underpowered to detect smaller effects, it is unlikely to reflect false positive data. In addition, it is important to note the current study used an *a priori* hypothesis and that reported associations are in line with previous ones. However, the findings should be cautiously interpreted and require confirmation in a larger cohort. We believe that all genetic data should be interpreted in the context of an individual's ancestral background. More important, all risk factors should be considered in a complex multifactorial disease, such as ACL injuries, to inform susceptibility. Risk susceptibility is most likely a combination of the interaction between a variety of extrinsic and intrinsic risk factors, including genetics.

A finely balanced inflammatory response is required for remodeling of the ECM[30] and that genetic polymorphisms potentially affect the production of inflammatory cytokines[12, 31].

The specific identity of these biological key role players however still remains unknown, including the threshold number and the time course of when they are required to direct the remodeling process within tendon and ligament. Therefore, future research should focus on the identification and quantification of inflammatory factors and on their time courses in tendon and ligament injuries. This may provide insights for biology-based therapies, such as anti-cytokine antibodies or cytokine antagonists and the most effective treatment period. Another approach might be to target cells that are responsible for the production of inflammatory cytokines, such as macrophages.

The *in vitro* experiments used dermal fibroblasts of eight individuals. Although dermal fibroblasts might have similar characteristics as tenocytes or ligamentocytes, their function and exact composition differ, possibly influencing their response to stimuli. In addition, a tissue-specific culture model applying a tensile force is required to study the effect of polymorphisms on matrix remodeling in more detail. Future research should aim to increase the number of donors. The difference in sex distribution in the genetic association study is explained by the fact that females participate less frequently in pivoting sports and therefore males and females were both tested together and separately for potential genetic associations with susceptibility for ACL injury.

In conclusion, this study describes specific polymorphisms within the inflammatory pathway to modulate the synthesis and degradation of structural and fibril-associated ECM components and thereby potentially contributing to an increased susceptibility to ACL injuries. This provisional evidence improves our understanding of the underlying mechanism for the genetic susceptibility to ACL ruptures and might lead to early identification of individuals who are of increased susceptibility to ACL injury and the potential application of personalized preventive or therapeutic interventions.

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SUPPLEMENTARY FIGURES AND TABLES

Supplementary Table 1. Primers used for RT-PCR analysis.

Gene	Forward primer (5' à 3')	Reverse primer (5' à 3')
COL5A1	GACAAGAAGTCCGAAGGGGC	TAGGAGAGCAGTTTCCCACG
COL1A1	TGAAGGGACACAGAGGTTTCAG	GTAGCACCATCATTTCCACGA
DCN	CAGACCAAGCACGCAAAACA	TCACAACCAGGGAACCTTGC
BGN	CACCGGACAGATAGACGTGC	CATGGCGGATGGACCTGGAG
IL6	GGATTCAATGAGGAGACTTGCC	GGGTCAGGGGTGGTTATTGC
IL1B	TTGCTCAAGTGTCTGAAGCAGC	CTTGCTGTAGTGGTGGTCGG
IL6R	CACGCCTTGGACAGAATCCA	TCCAGCAACCAGGAATGTGG
IL1R1	GGTAGACGCACCCTCTGAAG	GCATTTATCAGCCTCCAGAGAAGA
TNFRSFA1	ATTGGACTGGTCCCTCACCT	GTAGGTTCCTTTGTGGCACTT
CFL1	ATAAGGACTGCCGCTATGCC	CGGGGGCCCAGAAGATAAAC

Supplementary Table 2. Genetic risk profiles of the participants based on their (A) *IL1B* rs16944 C>T and (B) *IL6* rs1800795 G>C genotypes.

A.	Participant ID	*IL1B* rs16944	Sex
Low risk	5	CT	F
	7	CT	M
	8	CT	F
	11	CT	M
	22	TT	M
High risk	3	CC	M
	10	CC	M
	24	CC	F

B.	Participant ID	*IL6* rs1800795	Sex
Low risk	3	CC	M
	5	GC	F
	22	GC	M
	24	GC	F
High risk	7	GG	M
	8	GG	F
	10	GG	M
	11	GG	M

M, male; F, female.

Supplementary Table 3. Patient characteristics of the control (SWE-CON) group and the anterior cruciate ligament group with a noncontact (SWE-NON) mechanism of injury in a Swedish cohort[a].

	SWE-CON (n=116)	**SWE-NON** (n=79)	**p-value**
Age (years)[b]	44.7 ± 11.9 (114)	36.5 ± 13.7 (78)	**<0.001**
Sex (% male)	34.5 (116)	54.4 (79)	**0.014**
Height (cm)	172.3 ± 10.1 (108)	173.4 ± 8.6 (71)	0.438
Body mass (kg)[b]	72.1 ± 13.6 (107)	75.0 ± 12.8 (71)	0.149
Body mass index (kg/m^2)[b]	24.4 ± 2.9 (107)	24.7 ± 2.9 (70)	0.466

[a]*Values are presented as mean ± standard deviations except for sex, which is expressed as a percentage. The number of participants (n) with available data for each variable is in parenthesis.*

[b]*Self-reported values at the time of recruitment for the SWE-CON group, and at time of ACL rupture for the SWE-NON group.*

P-values in bold typeset indicate significance (p< 0.050).

Supplementary Table 4. Medical history and family injury for the control (SWE-CON) and non-contact (SWE-NON) anterior cruciate ligament rupture group of the Swedish cohort[a].

	Male			Female			
	SWE-CON (n=40)	SWE-NON (n=42)	p-value[b]	SWE-CON (n=76)	SWE-NON (n=37)	p-value[b]	p-value[c]
Previous ligament injury	88.6 (35)	100.0 (35)	0.114	73.5 (68)	81.8 (33)	0.458	**0.035**
Previous joint injury	35.1 (37)	51.3 (39)	0.235	37.5 (72)	41.7 (36)	0.834	0.230
Family history of ACL injury	15.2 (33)	24.3 (37)	0.384	17.4 (69)	33.3 (33)	0.121	0.093
• Grandparent	0.0 (33)	0.0 (37)	-	0.0 (69)	3.0 (33)	0.323	0.407
• Parent	0.0 (33)	2.7 (37)	1.000	7.2 (69)	12.1 (33)	0.466	0.531
• Sibling	12.1 (33)	10.8 (37)	1.000	2.9 (69)	6.1 (33)	0.593	0.551
• Child	3.0 (33)	10.8 (37)	0.361	7.2 (69)	6.1 (33)	1.000	0.551
• Other	0.0 (33)	0.0 (37)	-	0.0 (69)	6.1 (33)	0.103	0.164
Family history of joint injury	59.5 (37)	69.2 (39)	0.516	47.2 (72)	72.2 (36)	**0.024**	**0.014**
• Parent	21.6 (37)	46.2 (39)	**0.031**	31.9 (72)	47.2 (36)	0.181	**0.018**
• Sibling	43.2 (37)	48.7 (39)	0.804	15.3 (72)	44.4 (36)	**0.002**	**0.003**
• Child	27.0 (37)	17.9 (39)	0.415	20.8 (72)	19.4 (36)	1.000	0.608

[a]Values are expressed as percentages with the number of participants (n) with available data in parentheses.
[b]SWE-CON vs. SWE -NON, p-values in bold typeset indicate significance (p< 0.050).
[c]SWE-CON (male + female) vs. SWE-NON (male + female).
P-values in bold typeset indicate significance (p< 0.050).

Supplementary Table 5. Genotype effects per patient characteristic in the Swedish cohort.

A. Genotype effects *COL5A1* rs2228145 C>T.

	C/C (n=42)	**C/T** (n=83)	**T/T** (n=61)	**p-value**
Age (years)	40.2 ± 12.6 (40)	39.9 ± 13.2 (83)	44.0 ± 13.5 (60)	0.145
Sex (% male)	50.0 (21)	43.3 (36)	34.4 (21)	0.197
Height (cm)	174.7 ± 11.0 (38)	172.1 ± 9.0 (78)	171.9 ± 8.6 (55)	0.308
Body mass (kg)	74.1 ± 11.8 (38)	77.3 ± 13.8 (77)	73.3 ± 13.2 (55)	0.782
Body mass index (kg/m^2)	24.0 ± 2.7 (37)	24.6 ± 2.8 (77)	24.7 ± 3.1 (55)	0.536

B. Genotype effects *IL1B* rs16944 C>T.

	C/C (n=78)	**C/T** (n=77)	**T/T** (n=35)	**p-value**
Age (years)	42.2 ± 13.2 (77)	39.8 ± 13.3 (75)	42.2 ± 13.5 (35)	0.483
Sex (% male)	50.0 (39)	31.1 (24)	48.6 (17)	0.091
Height (cm)	173.8 ± 9.3 (72)	171.2 ± 9.1 (72)	173.7 ± 9.5 (30)	0.203
Body mass (kg)	75.7 ± 12.2 (70)	71.6 ± 12.8 (72)	73.0 ± 16.0 (31)	0.172
Body mass index (kg/m^2)	24.9 ± 2.8 (70)	24.3 ± 2.7 (72)	24.7 ± 3.2 (30)	0.360

C. Genotype effects *IL6* rs1800795 G>C.

	C/C (n=45)	**C/G** (n=107)	**G/G** (n=38)	**p-value**
Age (years)	44.8 ± 12.5 (38)	40.8 ± 13.7 (104)	40.2 ± 12.7 (45)	0.211
Sex (% male)	47.4 (18)	37.4 (40)	46.7 (21)	0.536
Height (cm)	172.7 ± 8.3 (36)	172.3 ± 9.7 (96)	173.0 ± 9.7 (43)	0.927
Body mass (kg)	73.1 ± 11.5 (72)	73.5 ± 14.6 (96)	73.1 ± 12.1 (43)	0.983
Body mass index (kg/m^2)	24.5 ± 2.6 (35)	24.7 ± 3.0 (95)	24.3 ± 2.7 (43)	0.679

D. Genotype effects *IL6R* rs2228145 A>C.

	A/A *(n=94)*	**A/C** *(n=76)*	**C/C** (n=18)	**p-value**
Age (years)	41.4 ± 13.2 (93)	40.9 ± 14.2 (75)	42.9 ± 8.0 (17)	0.850
Sex (% male)	42.6 (40)	36.8 (28)	55.6 (10)	0.882
Height (cm)	172.2 ± 9.7 (84)	172.5 ± 8.6 (70)	174.6 ± 9.6 (18)	0.610
Body mass (kg)	72.4 ± 11.7 (85)	73.5 ± 14.4 (69)	75.4 ± 12.4 (17)	0.653
Body mass index (kg/m^2)	24.2 ± 2.6 (84)	24.8 ± 3.0 (69)	24.7 ± 2.6 (17)	0.407

Supplementary Table 6. Genotype and minor allele frequency distributions, and p-values for Hardy-Weinberg exact test of the four selected polymorphisms within the South African (SA) and Swedish (SWE) cohort for asymptomatic controls (CON), the anterior cruciate ligament (ACL) rupture group and the ACL subgroup with a noncontact (NON) mechanism[a].

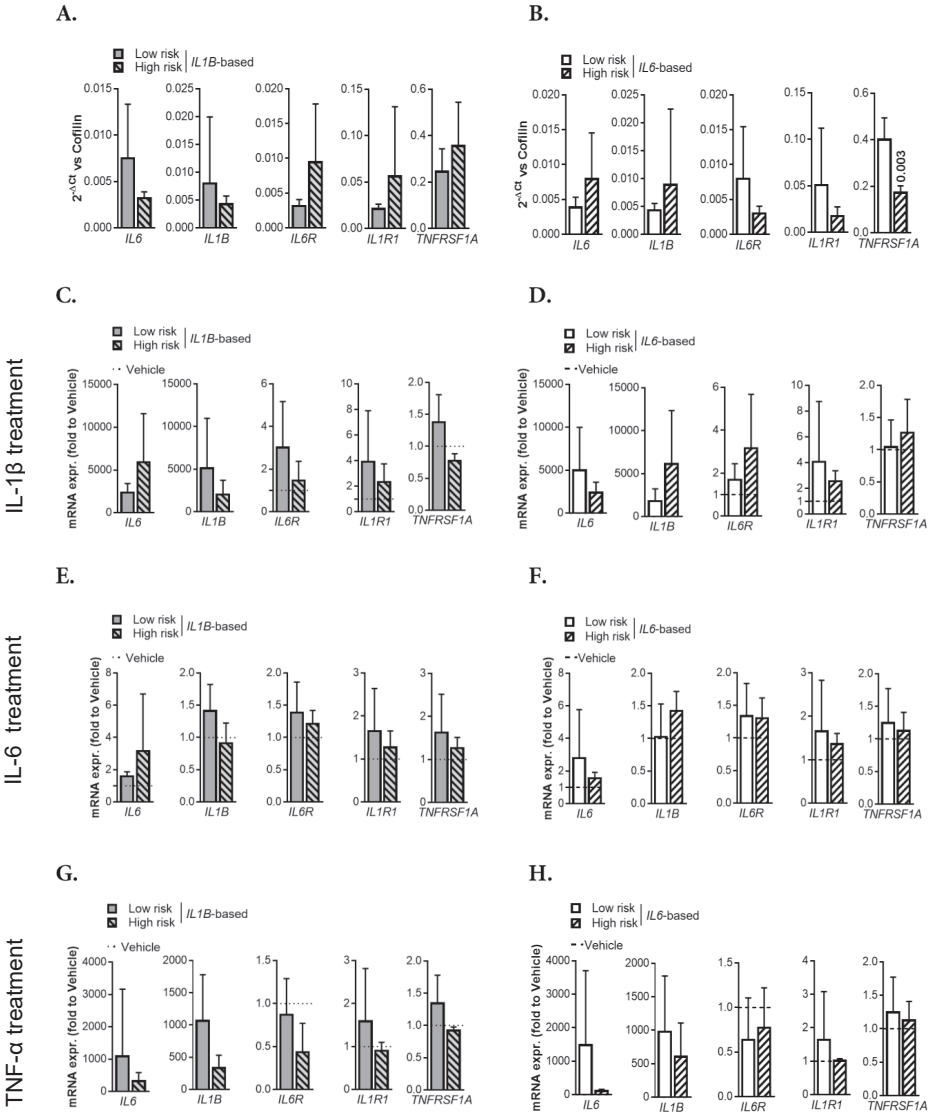
		CON			ACL				
		SA-CON	SWE-CON	P-value[b]	SA -ACL	SWE -ACL	P-value[b]	SA -NON	P-value[c]
	n	*96*	*109*		*93*	*71*		*48*	
	CC	38 (36)	22 (24)	**<0.001[d]**	41 (38)	23 (18)	**0.019[d]**	35 (17)	**0.021[d]**
COL5A1 **rs12722 C > T**	CT	49 (47)	43 (47)		45 (42)	47 (36)		54 (26)	
	TT	14 (13)	35 (38)		14 (13)	30 (23)		10 (5)	
	T allele	38 (73)	56 (123)	**<0.001[d]**	37 (68)	53 (82)	**0.003[d]**	38 (36)	**0.022[d]**
	HWE	0.829	0.241		0.824	0.648		0.367	
	n	*93*	*112*		*93*	*78*		*48*	
	CC	24 (24)	39 (44)	**0.033[d]**	27 (25)	44 (34)	**0.033[d]**	27 (13)	0.095
IL1B **rs16944 C > T**	CT	47 (46)	41 (46)		52 (48)	40 (31)		44 (21)	
	TT	29 (28)	20 (22)		22 (20)	17 (13)		29 (14)	
	T allele	52 (102)	40 (90)	**0.019[d]**	47 (88)	37 (57)	0.058	51 (49)	**0.033[d]**
	HWE	0.549	0.120		0.836	0.224		0.395	
	n	*98*	*113*		*98*	*77*		51	
	GG	72 (71)	22 (25)	**<0.001[d]**	64 (63)	26 (20)	**<0.001[d]**	67 (34)	**<0.001[d]**
IL6 **rs1800795 G > C**	GC	26 (25)	59 (67)		31 (30)	52 (40)		27 (14)	
	CC	2 (2)	19 (21)		5 (5)	22 (17)		6 (3)	
	C allele	15 (29)	48 (109)	**<0.001**	20 (40)	48 (74)	**<0.001[d]**	20 (20)	**<0.001[d]**
	HWE	1.000	0.061		0.539	0.821		0.373	
	n	*112*	*95*		*76*	*95*		49	
	AA	53 (59)	54 (51)	0.996	46 (35)	58 (55)	0.165	55 (27)	0.886
IL6R **rs2228145 A > C**	AC	37 (41)	34 (32)		46 (35)	39 (37)		43 (21)	
	CC	11 (12)	13 (12)		8 (6)	3 (3)		2 (1)	
	C allele	29 (65)	29 (56)	1.000	31 (47)	23 (43)	0.108	23 (23)	0.256
	HWE	0.082	0.253		0.385	0.599		0.257	

[a]Genotype and allele frequencies are expressed as a percentage with the number of participants (n) in parentheses.

[b]SA vs. SWE cohort (unadjusted p-value).

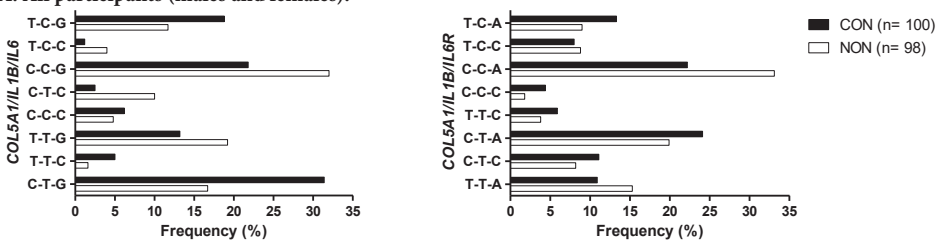
[c]SA with non-contact mechanism vs. SWE cohort (unadjusted p-value).

[d]P-values in bold typeset indicate significance (p< 0.050).

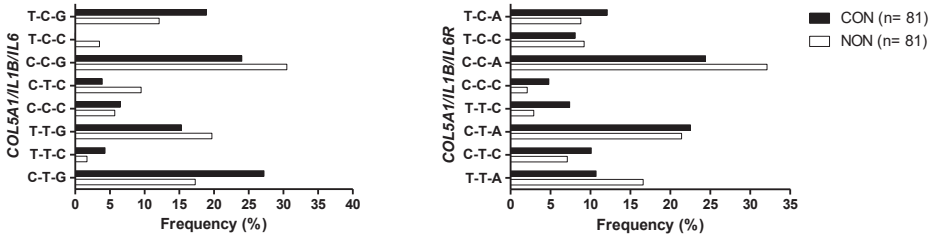


Supplementary Figure 1: mRNA expression in unstimulated fibroblasts (A, B) and fibroblasts stimulated with IL-1β (C, D), IL-6 (E, F) or TNF-α (G, H). mRNA expression levels of the cytokine-related genes, IL6, IL1B, IL6R, IL1R1 and TNFRSF1AR in fibroblasts classified in high-risk or low-risk for ACL injuries based on (A) *IL6* rs1800795 G>C or (B) *IL1B* rs12722 C>T. Data is presented as $2^{-\Delta Ct}$ to assess gene expression compared to CFL1 (housekeeping gene). Data is presented as mean with standard deviation (SD). Unpaired two-tailed Student's *t*-test, P-values in bold indicate significance (p<0.050).

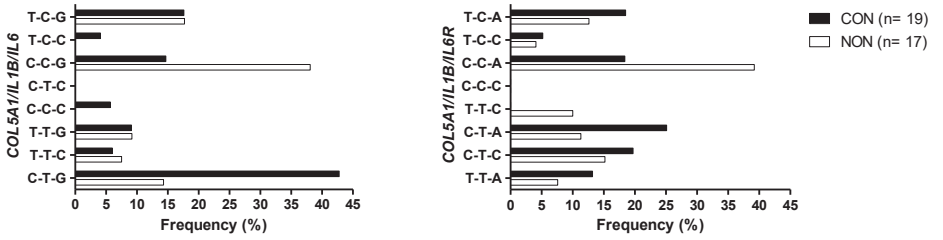
A. All participants (males and females).



B. Male participants.

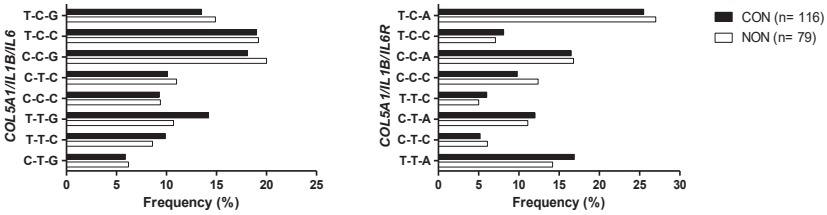


C. Female participants.

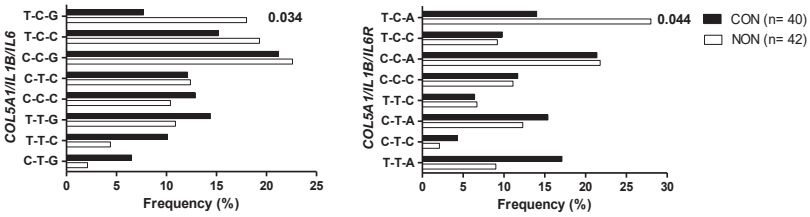


Supplementary Figure 2: Frequency distributions in the South African cohort for the *COL5A1* rs12722 C>T, *IL1B* rs16944 C>T, *IL6* rs1800795 G>C or *IL6R* rs2228145 A>C polymorphisms in the control group (CON; black bars) and the anterior cruciate ligament rupture group (ACL; white bars) for (A) all participants (males and females), (B) the male participants and (C) female participants. The number of participants (n) in each group is in parentheses.

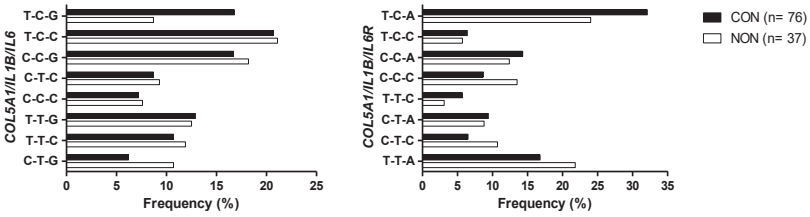
A. All participants (males and females).



B. Male participants.



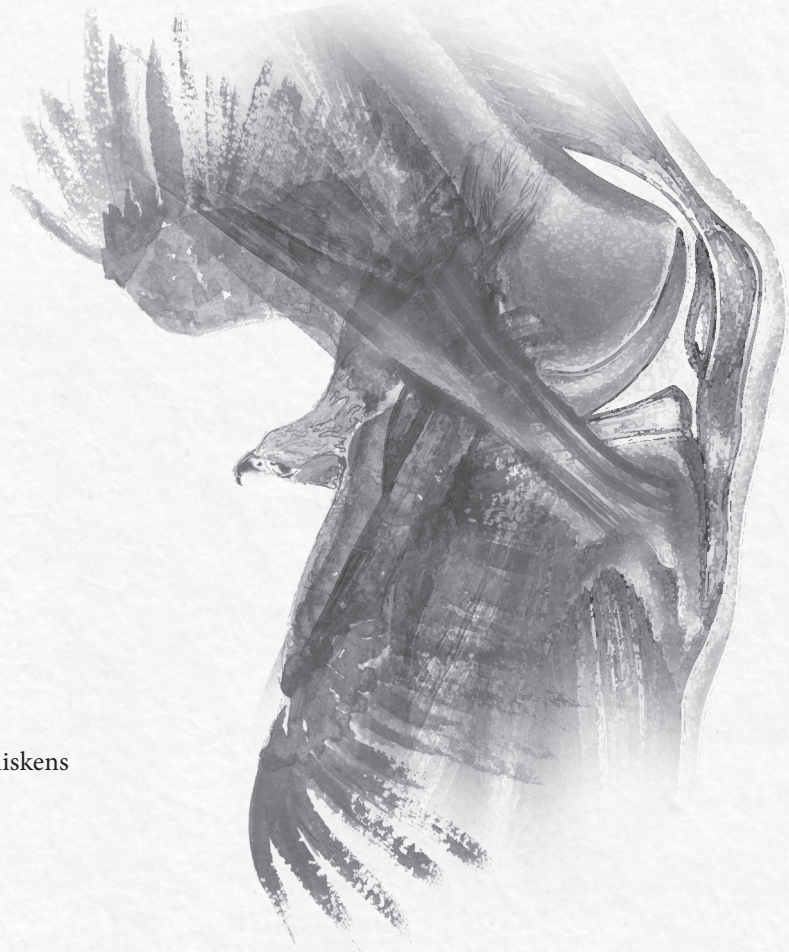
C. Female participants.



Supplementary Figure 3: Frequency distributions in the Swedish (SWE) cohort for the *COL5A1* rs12722 C>T, *IL1B* rs16944 C>T, *IL6* rs1800795 G>C or *IL6R* rs2228145 A>C polymorphisms in the control group (SWE-CON; black bars) and the non-contact anterior cruciate ligament rupture group (SWE-NON; white bars) for (A) all participants (males and females), (B) male participants and (C) female participants in the Swedish cohort. The number of participants (n) in each group is in parentheses. P-values in bold typeset indicate significance (p< 0.050).

CHAPTER 6

Inhibiting phosphorylation of STAT proteins modulates the inflammatory phenotype of osteoarthritic synovium



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ABSTRACT

Synovial inflammation plays an important role in the pathological process of osteoarthritis (OA). This study determines phosphorylation levels of signal transducer and activator of transcription (STAT) proteins in osteoarthritic synovium and investigates whether inhibition of STAT signaling pathways modulates the inflammatory phenotype of OA synovium. To examine this, STAT1, 3, and 6 phosphorylation were determined in OA synovium using Western blot analysis. This was done either directly after harvest or after treatment with 50 μM NSC118-218, 100 μM S3I-201, or 100 nM AS1517499, compounds known to inhibit STAT phosphorylation, for 24 hours with or without the presence of synovial fluid. Different STAT1 and STAT3 phosphorylation levels were observed in OA synovium among the donors. Phosphorylated STAT6 was only detectable when explants were cultured in synovial fluid. NSC118-218 and AS1517499 inhibited STAT1 phosphorylation, although only treatment with AS1517499 also resulted in decreased *IL1B* and *IL6* gene expression. S3I-201 inhibited STAT3 phosphorylation, resulting in less *IL6* expression and increased *TNFA* expression. These data indicate that donor dependent STAT1, 3, and 6 phosphorylation patterns are found in OA synovium. Inhibition of STAT1 phosphorylation had an anti-inflammatory effect, whereas the inhibition of STAT3 phosphorylation enhanced the inflammatory phenotype. Inhibition of STAT1 phosphorylation might be a potential therapy to diminish synovial inflammation eventually to slow down or prevent the pathogenesis of OA.

Key words*:* Inflammation; modulation; JAK-STAT signalling; osteoarthritis; synovium.

INTRODUCTION

Inflammation of the synovial membrane is a common feature of osteoarthritis (OA) with an accumulation of infiltrating immune cells, such as macrophages[10]. Cells within the inflamed synovial membrane produce factors known to have catabolic effects, such as interleukin (IL) 1β and IL6[29, 35]. The pathophysiological process that occurs in the osteoarthritic joint is largely mediated by pro-inflammatory cytokines and other mediators[35]. However, OA also results in elevated levels of the anti-inflammatory cytokines IL4 and IL10 in the serum or synovial fluid[16, 22, 33]. Therefore, a targeted approach is required to specifically modulate the inflammatory phenotype of the synovial membrane. The intracellular Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathways are a principal signaling mechanism for a wide array of cytokines and growth factors. Signaling through this pathway is mediated by phosphorylation of STAT proteins. It is known that when cells, including fibroblasts and macrophages, are stimulated with interferon (IFN) γ, STAT 1 is phosphorylated[17, 19]. Similarly, IL4 in general results in activation of STAT6, whereas stimulation with IL10 leads to activation of STAT3[1, 25]. Once phosphorylated, STAT proteins form dimers and subsequently regulate gene expression[8]. More specifically, phosphorylated STAT1 regulates transcription of *IL1B* [19], whereas phosphorylated STAT3 targets the *IL6* and *IL10* genes[11, 15]. The anti-inflammatory gene *IL4* is targeted by phosphorylated STAT6[18]. Although previously investigated in rheumatoid arthritis, it is currently unknown which JAK-STAT pathways are activated in OA synovial tissue and whether modulation of JAK-STAT pathways affects the inflammatory phenotype of the synovium.

The main cell types in synovial explants are fibroblasts and macrophages. Macrophages can become activated by environmental cues, resulting in different phenotypes ranging from pro-inflammatory to anti-inflammatory or repair macrophages. *In vitro*, pro-inflammatory or M1-like macrophages can be obtained by stimulation with IFNγ and/or TNFα[4] among others. Macrophages induced by stimuli such as IL4 or IL13 obtain a repair-phenotype and are often referred to as M2-like macrophages. M2-like macrophages induced with IL10 or glucocorticoids obtain a predominant anti-inflammatory phenotype[27]. These subtypes represent the extremes of a spectrum and are a simplified version of the range of phenotypes that can appear *in vivo*. As macrophages are one of the sources for pro-inflammatory cytokines in the knee joint during OA, macrophages were completely depleted from the knee joint prior to OA induction. Indeed, macrophages contributed to the onset and progression of osteoarthritis[31, 32]. However, depleting all synovial macrophages is a non-specific approach and might therefore also abolish some of their beneficial effects. Another approach is to neutralize specific cytokines, but this did not seem to be completely effective in every patient suffering from OA[5]. Targeting proteins involved in intracellular signal transduction pathways might therefore be

an interesting new strategy as it is more specific than depleting cells and it affects the production of multiple cytokines at once.
The aim of the present study was to determine STAT activation in OA synovium and to investigate whether inhibiting STAT signaling pathways modulates the inflammatory phenotype of osteoarthritic synovium. In addition, as macrophages are key role players in inflammation, we determined presence of macrophage phenotypes in OA synovium and their respective STAT activation levels.

METHODS

Modulating synovial tissue

Synovial tissue was obtained from 17 patients with gonarthrosis undergoing total knee replacement at Erasmus MC, University Medical Center, Rotterdam. Consent was given in accordance with the guidelines of the Federation of Biomedical Scientific Societies (www.federa.org) after approval by the local ethical committee (#MEC2004-322). The synovium was washed twice with 0.9% NaCl (Sigma-Aldrich, Saint Louis, Missouri, USA), separated from the surrounding tissue and cut in to 5mm^2 pieces.
To examine initial activation of STAT proteins, synovial explants were not cultured, but immediately stored at -80°C (n=4, male: 1, mean age: 64). To inhibit STAT phosphorylation, explants were cultured for 24 hours with or without 50μM NSC118-218, 100 μM S3I-201 and 100 nM AS1517499 in medium (Dulbecco's Modified Eagle Medium, low glucose (DMEM; Gibco, Carlsbad, USA) complemented with 10% FCS (n=5, male: 2, mean age: 61) with or without additional 50% synovial fluid from the same donor as the synovial tissue was obtained (n=4, male: 3, mean age: 79). After the culture period, synovial explants were harvested and stored at -80°C until evaluation using Western Blot analysis and gene expression analysis. The medium was harvested and stored at -80°C for cytokine measurements. Doses were chosen based on the current literature. Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used as vehicle for all inhibitors with a final DMSO concentration in cultures of <0.01%.

RNA isolation and quantitative RT-PCR

Frozen synovial samples were processed using a Mikro-Dismembrator S (B. Braun Biotech International GmbH, Melsungen, Germany) and consecutively samples were dissolved in 350μl Trizol. A RNeasy Micro Kit (QIAGEN, Venlo, The Netherlands) was used to extract RNA and procedures were performed according to the manufacturer's instructions. RNA was quantified using Nanodrop ND-1000 Spectrophotometer (NanoDrop ND1000 UV-VIS, Isogen Life Science B.V., the Netherlands). A total of 250 ng RNA per sample was reversed transcribed into cDNA using RevertAidTM First strand cDNA synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Quantitative real-time PCR was performed on an ABI

Prism 7000 Sequence detection system (Applied Biosystems, Foster City, Ca) using either TaqMan Universal PCR mastermix (Applied Biosystems) or SybrGreen (Eurogentec). Gene expression of *IL1B*, *TNFA*, *CCL18*, Cluster of Differentiation 206 (*CD206*) and *CD163* was evaluated. Glyceraldehyde-3-phosphate Dehydrogenase (*GAPDH*), Hypoxanthine Phosphoribosyltransferase 1 (*HPRT1*) and Beta-2-microglobulin (*B2M*) were all tested as housekeepers, where *GAPDH* was found the most stable (data not shown) and was therefore further used as normalization for the genes of interest. Relative quantification of PCR signals was performed by comparing the threshold cycle value (C_t) for the gene of interest in each sample with the C_t value for the housekeeping gene[23].

Quantification of cytokine production

Quantification of IL6, CCL18 and soluble CD163 (sCD163) in the medium of synovium cultures was performed by enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions of human IL6 ELISA Development Kit (PeproTech), human CCL18 DuoSet Development Kit (R&D Systems) and human soluble CD163 DuoSet Development Kit (R&D) systems.

Western blotting

Sample's protein concentrations were measured using a bichinchoninic acid assay (BCA assay) according to the manufacturer's instructions (Thermo Scientific). For immunoblot detection of phosphorylated STAT proteins, 10 – 20 µg of the samples were electrophoresed on 10% SDS-polyacrylamide gels (Thermo Scientific, or Invitrogen, Carlsbad, CA USA), electrotransferred onto nitrocellulose membranes and blocked with 5% non-fat dried milk for two hours. Membranes were incubated overnight at 4°C with primary antibodies against phosphorylated STAT1 (Cell Signaling Technology, Danvers, MA, USA), phosphorylated STAT3 (Cell Signaling Technology), phosphorylated STAT6 (Cell Signaling Technology) and α-Tubulin (Cell Signaling Technology).
After washing, the membranes were incubated with horseradisch peroxidase (HRP) –conjugated anti-rabbit IgG secondary antibody (Cell Signaling) for 1.5 hour at room temperature. Antibody detection was performed using SuperSignal West Pico Luminol Enhancer Solution and SuperSignal West Pico Stable Peroxide Solution (Thermo scientific). Western blot analyses were quantified using Image J (U.S. National Institutes of Health, Bethesda, Maryland, USA).

Immunohistological analysis

Synovial samples from 3 different donors (n=3, male: 1, mean age: 70 years) were collected and 6 µm thick cryosections were cut. Sections were fixed in acetone for 10 minutes and subsequently washed with PBS. Following blocking with 10% goat serum (Southern Biotech #0060-01) for 30 minutes, sections were incubated for 1 hour at room temperature

with an antibody against CD68 (Abcam, clone KP-1 ready-to-use) as pan-macrophage marker, CD86 (Genetex, clone EP1158Y; 0.45 μg/ml) as M1 marker, CD206 (Abcam, #64693; 2.5 μg/ml) as M2a marker, and CD163 (Abcam, #182422; 1.6 μg/ml) as M2c marker. Sections were incubated for 30 minutes with either second antibody biotinylated goat-anti-mouse Ig link (BioGenex, HK-325-UM) or a biotinylated goat-anti-rabbit Ig link (Biogenex, HK-326-UR) diluted with PBS/1%BSA. This was followed by incubation with a third antibody: alkaline phosphatase-conjugated streptavidin label diluted 1:50 in PBS/1%BSA (BioGenex, HK-321-UK). After a final wash step in Tris-HCl, sections were incubated in freshly prepared substrate mixture of Neu Fuchsin (Chroma Gesellschaft, 1g/25ml 2M HCl), Sodiumnitrate (Sigma, #S2252) and Naphtol AS-MX phosphate (Sigma, #N5000) that was dissolved in di-methylformamid (Sigma, D4551). Subsequently tissue sections were counterstained with Haematoxylin Gill's (Sigma, #GHS232) to evaluate the overall staining pattern better. An isotype-matched control antibody; either monoclonal mouse IgG1 (Dako Cytomation #X0931) or rabbit IgG1 antibody (Dako Cytomation #X0903) was used as negative control for each staining. Sections were dried overnight and mounted with VectaMount (Vector Laboratories, #H5000).

Human monocyte isolation and culture

Monocytes were isolated from human buffy coats of healthy male donors (Sanquin Blood bank, Amsterdam, the Netherlands) by Ficoll density gradient separation and $CD14^+$ selection as described before[14]. Isolated monocytes plated in 24-well plates (Corning Incorporated, NY, USA) at a density of 500,000 cells/cm^2 in X-VIVO 15 medium (Lonza, Verviers, Belgium) supplemented with 20% heat-inactivated fetal calf serum (FCS, Lonza), 50 μg/mL gentamycin and 1.5 μg/mL fungizone. Monocytes were differentiated towards different macrophage phenotypes using 10 ng/mL IFNγ (Peprotech, Rocky Hill, NJ, USA) and 10 ng/mL TNFα (Peprotech) to obtain M(IFNγ + TNFα), 10 ng/mL IL4 (Peprotech) to obtain M(IL4) or 10 ng/mL IL10 (Peprotech) to obtain M(IL10).
Activated macrophages were harvested 30, 60, and 90 minutes after plating. These cells were suspended in 180 μL M-PER (Thermo Scientific, Rockford, USA) with 0.1% HALT™ – Protease Inhibitor Single-Use Cocktail (100x) (Thermo Scientific) and 0.1% Phosphatase Inhibitor (Thermo Scienitific) and stored at -80°C until Western Blot analysis was performed. After 24 hours, the medium of macrophages was collected, centrifuged at 200x g and the supernatants were stored at -80°C for cytokine measurement. Harvested cells were resuspended in PBS/0.1% Triton X-100 (Sigma-Aldrich, St. Louis, USA) for DNA quantification or in TRIzol Reagent (Ambion, Carisbad, CA, USA) for mRNA isolation.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistics for Windows (version 21.0, IBM Corp., Armonk, NY). A mixed linear model with a Bonferroni post-hoc test

was used after log transformation to statistically analyze the data and to take into account donor variability between different donors. In all experiments, an individual experiment was considered as a random factor. For macrophages cultured in monolayers, polarization states were considered as a random factor. In the experiments with synovial explants, treatment with an inhibitor was considered as a random factor.
Differences were considered to be statistically significant if $p < 0.05$.

RESULTS

STAT phosphorylation in end-stage OA synovium

Phosphorylated STAT1 was detectable in synovial explants obtained immediately after surgery in 3 of the 5 OA donors evaluated. pSTAT3 could be detected in synovial tissue of all donors, although the levels of expression seem variable. No pSTAT6 could be detected in the collected synovial explants, only when the explants were stimulated with IL4 (as a control) (Figure 1).

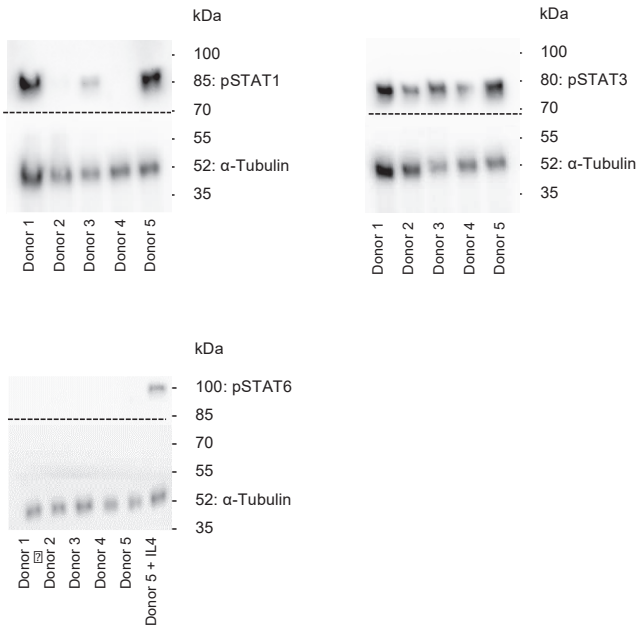


Figure 1. Presence of phosphorylated STATs in OA synovial tissue.
Western Blots analysis of OA synovial tissue in 5 donors. The dotted line indicates grouping of images from different parts of the same gel.

Therefore, presence of phosphorylated STATs was examined in explants that were cultured in synovial fluid (SF). Indeed, in the presence of synovial fluid, pSTAT6 was detectable in all explants in variable degrees (Figures 2A, D and G), next to pSTAT1 and pSTAT3.

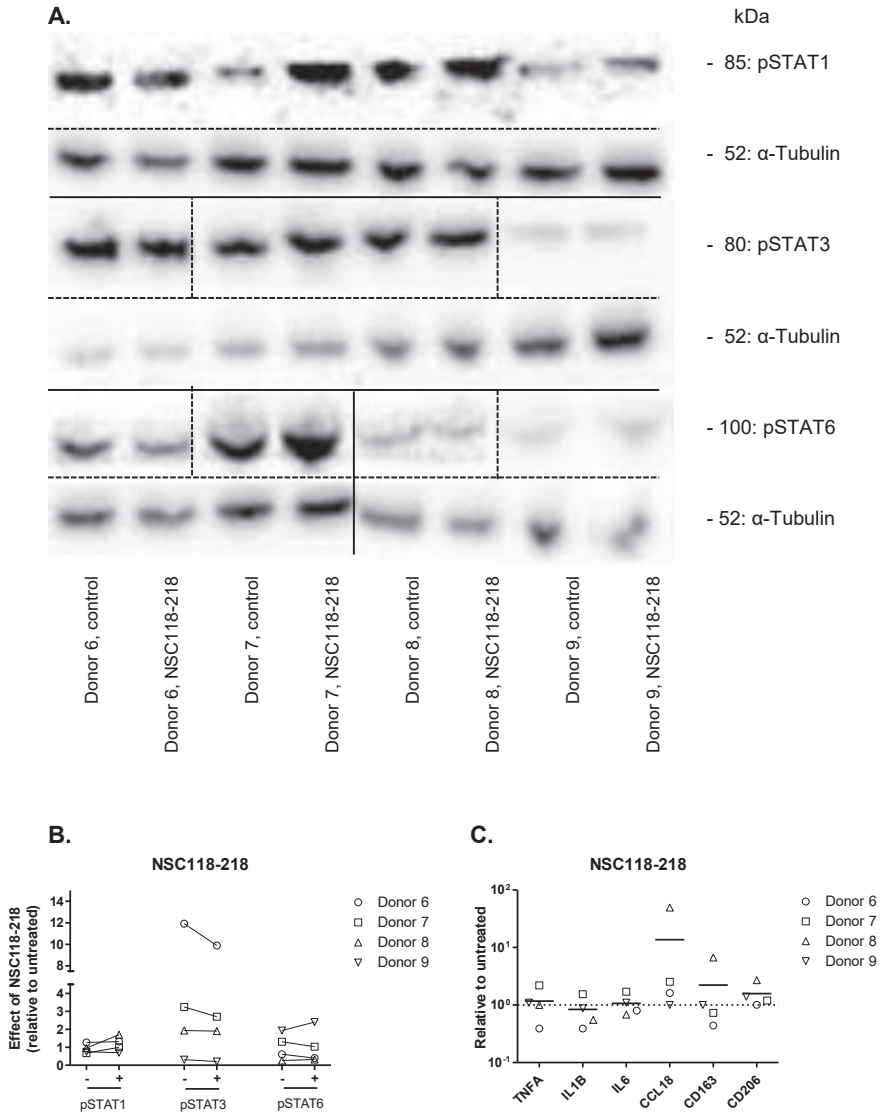


Figure 2. Modulation of STAT phosphorylation in OA synovial tissue cultured in synovial fluid.

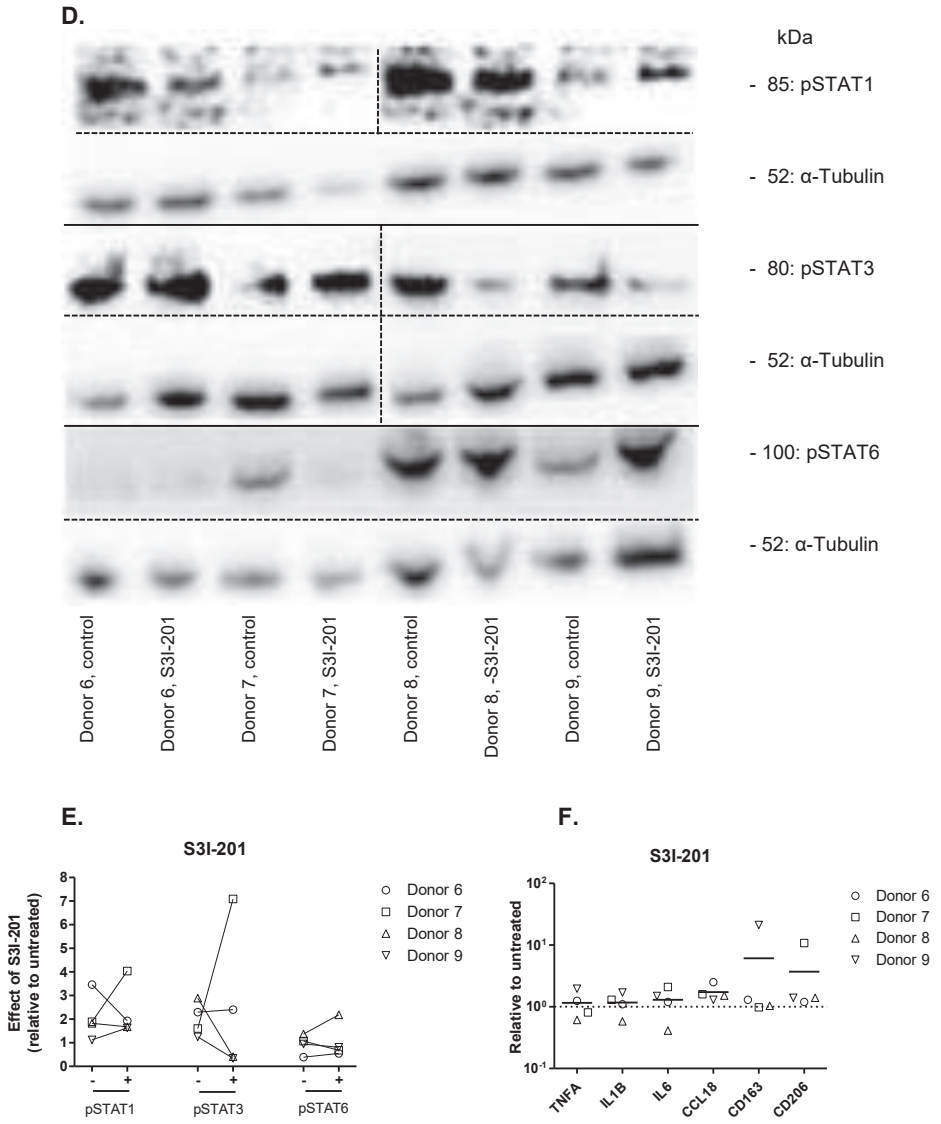


Figure 2. Modulation of STAT phosphorylation in OA synovial tissue cultured in synovial fluid.

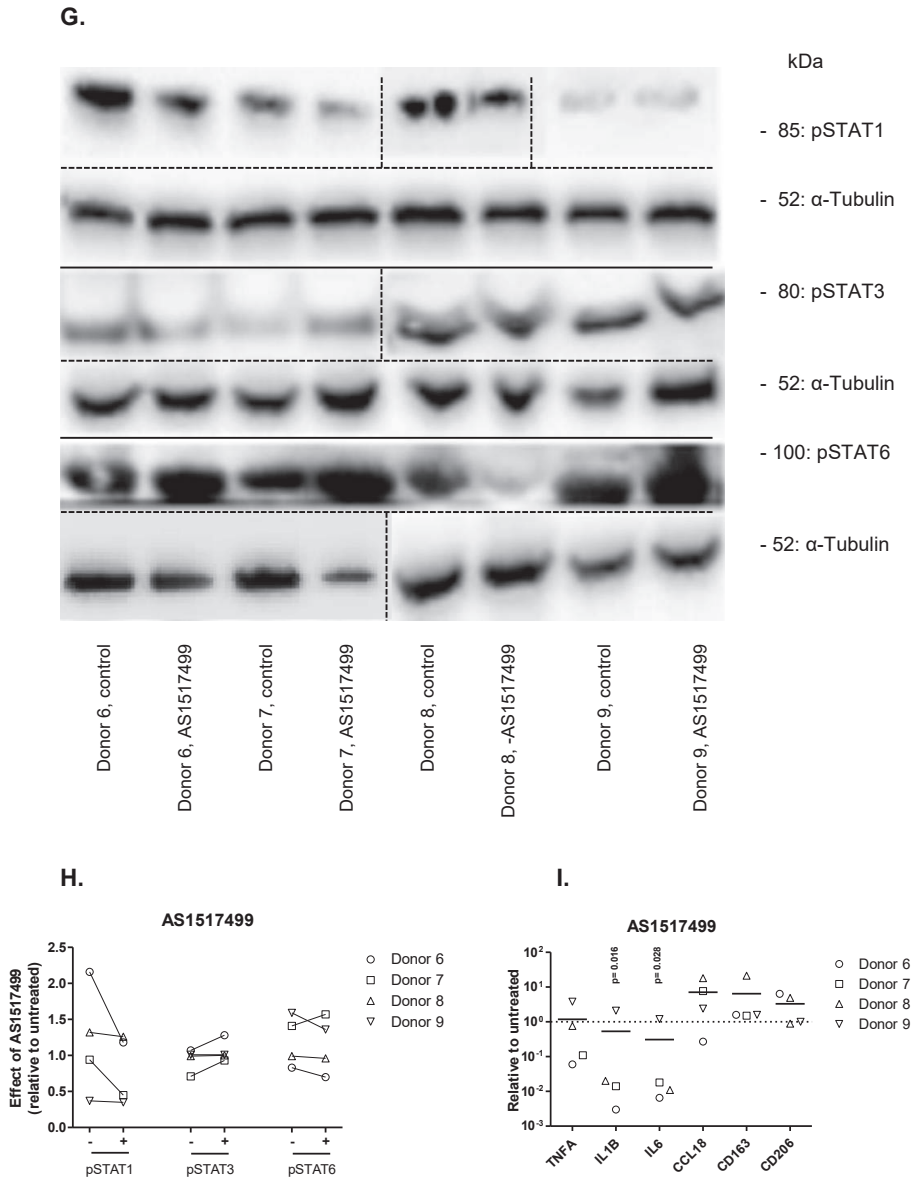


Figure 2. Modulation of STAT phosphorylation in OA synovial tissue cultured in synovial fluid.
A, D, G*) Western Blot analysis of synovial tissue after treatment with the STAT inhibitors. The dotted line indicates grouping of images from different parts of the same gel, whereas the continuous line indicates grouping of different parts from different gels. -; control, +; treated with STAT inhibitor.* ***B, E, H****) Quantification of the Western Blots relative to α Tubulin.* ***C, F, I****) Gene expression corrected for GAPDH and relative to the untreated control, which is represented by the dotted line. Data is shown as mean (as indicated by line) for n=4 analysed in 2-fold.*

Modulation of OA synovial tissue with STAT-inhibitors

After determining STAT phosphorylation in human OA synovium, the effect of STAT1, 3, and 6 inhibition on the inflammatory profile was assessed in the tissue. Since especially STAT6 phosphorylation seems only detectable in OA synovium when certain stimuli are present, we decided to culture all the explants in the donor's own SF. Culturing synovial explants with 50 μM NCS118-218, known as STAT1 inhibitor, did not affect STAT1 phosphorylation, nor did it affect pSTAT3 or pSTAT6 levels (Figure 2A and B). 100 μM S3I-201, known as STAT3 inhibitor, did not influence STAT3 phosphorylation in explants cultured in SF, nor did it affect STAT1 or STAT6 phosphorylation (Figure 2D and E). 100 nM AS1517499, chosen as STAT6 inhibitor, decreased STAT1 phosphorylation levels, without affecting pSTAT6 levels (Figure 2G and H). This decrease in STAT1 phosphorylation levels was accompanied by a significant decrease of *IL1B* and *IL6* levels (Figure 2I).

The effect of the STAT1 and 3 inhibitors was also tested in cultures without synovial fluid. Here, NSC-118218 did result in a significant decrease of pSTAT1 levels (Figure 3A and B), without affecting gene expression levels (Figure 3C) or secreted protein production (Figure 3D). Treatment of synovial explants with S3I-201 decreased phosphorylated STAT3 levels in 4 of the 5 donors, and increased pSTAT3 in one of the donors (Figure 3E and F). This treatment led to a significant upregulation of *TNFA* and a significant downregulation of *IL6* and *CD163* (Figure 3G). S3I-201 treatment also did not alter protein secretion after 24 hours of culture (Figure 3H). As pSTAT6 is not present at detectable levels in OA synovial tissue in absence of the corresponding environmental cues, STAT6 phosphorylation levels were not determined in synovial explants in culture medium nor was AS1517499 tested under these conditions.

Characterization of human macrophage phenotypes and their presence in OA synovial tissue

Using immunohistochemistry, the presence of macrophages was analysed in OA synovium. CD68 used as pan-macrophage marker, was mainly found in the lining of OA synovium explants. The presence of CD86, indicating pro-inflammatory macrophages, and CD206, indicating tissue repair macrophages, was mainly seen in the lining of OA synovium explants. CD163, a marker indicating anti-inflammatory cells, was present in the synovial lining of donor 1 and in the sublining of donor 2 and 3. Isotype controls were negative for all stainings (Figure 4A).

To investigate the activity of the different STATs related to different macrophage subtypes and cytokine expression, we stimulated macrophages towards specific phenotypes *in vitro*. pSTAT1 was abundant in M(IFNγ+TNFα) at all three time points after activation and not detectable in M(IL10) and M(IL4). pSTAT3 was highly present in M(IL10). pSTAT6 was only detected in M(IL4), but not in M(IFNγ+TNFα) or M(IL10), and phosphorylation

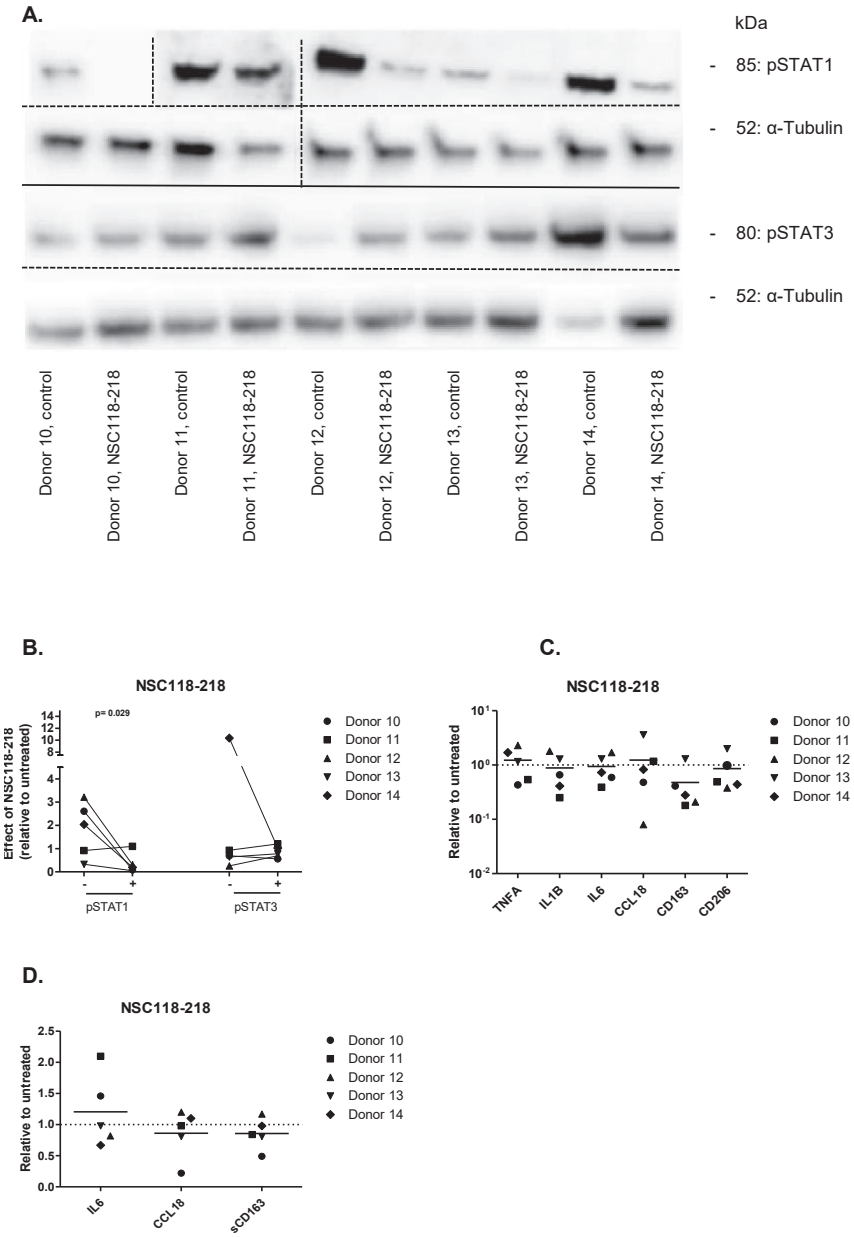


Figure 3. Modulation of STAT phosphorylation in OA synovial tissue cultured in medium.

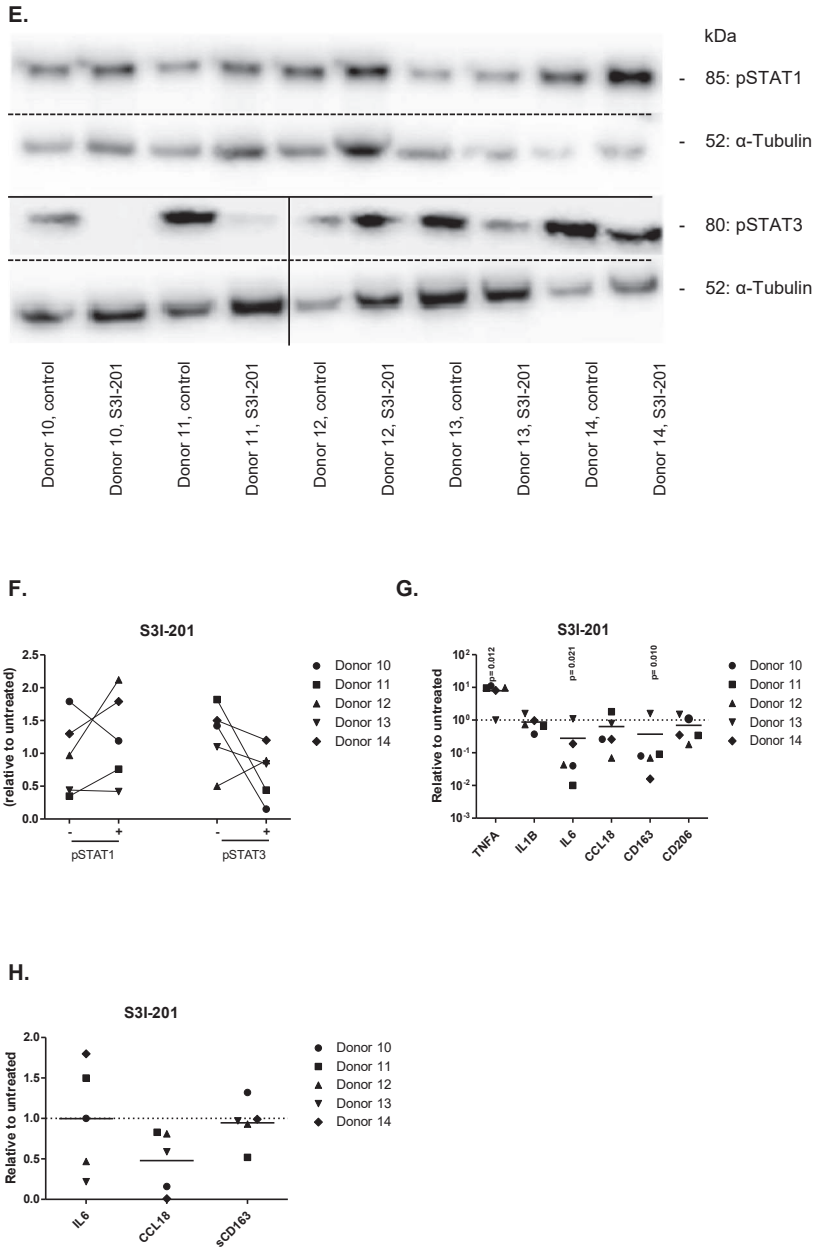


Figure 3. Modulation of STAT phosphorylation in OA synovial tissue cultured in medium.

A, E) *Western Blot analysis of synovial tissue after treatment with the STAT inhibitors. The dotted line indicates grouping of images from different parts of the same gel, whereas the continuous line indicates grouping of different parts from different gels. -; control, +; treated with STAT inhibitor.* ***B, F)*** *Quantification of the Western Blots relative to α Tubulin.* ***C, G)*** *Gene expression corrected for GAPDH and relative to the untreated control, which is represented by the dotted line.* ***D, H)*** *Protein production of IL6, CCL18 and sCD163. Data is shown as mean (as inidcated by the line) for n=5 donors analysed in 2-fold.*

rapidly decreased after stimulation (Figure 4B). M(IFNγ+TNFα) had significantly higher gene expression levels of *TNFA, IL6* and *IL1B*, than the other macrophage phenotypes. M(IL4) expressed the highest levels of *CCL18* and *CD206*. M(IL10) expressed significantly more *CD163* than M(IFNγ+TNFα) (Figure 4C). Although not significant, IL6 protein was the highest in culture medium of M(IFNγ+TNFα), CCL18 was the highest in M(IL4) and sCD163 in M(IL10) (Figure 4D).

To examine whether the analysed pSTATs in synovium are differentially activated in different macrophage phenotypes, we analysed pSTAT1, 3, and 6 in different *in vitro* obtained macrophage phenotypes. Samples were taken at different time points after stimulation since STAT activation can be very dynamic.

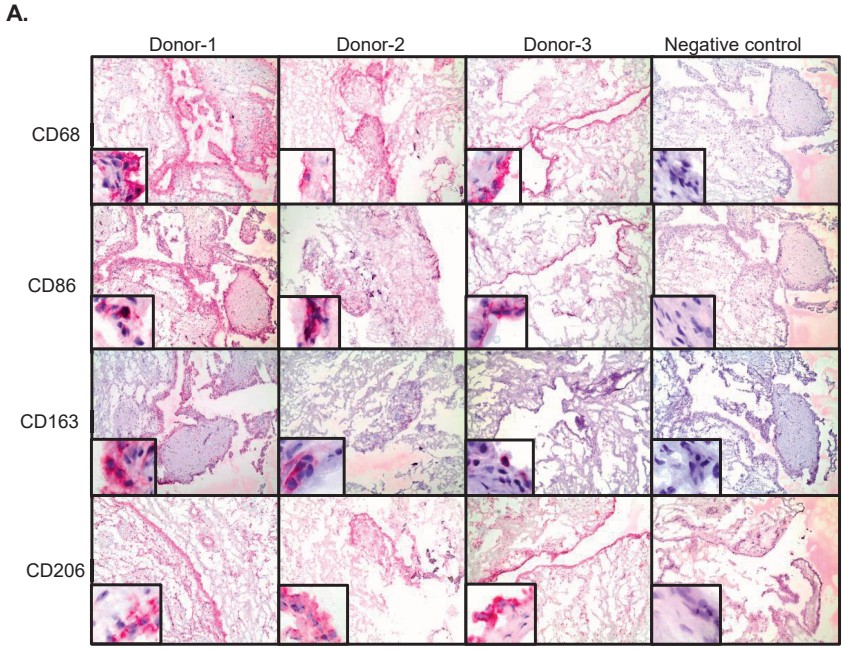


Figure 4. Differences in macrophage phenotypes in OA synovium and characterization of primary human macrophage phenotypes, stimulated with IFNγ and TNFα (M (IFNγ + TNFα)), IL4 (M(IL4)) or IL10 (M(IL10))

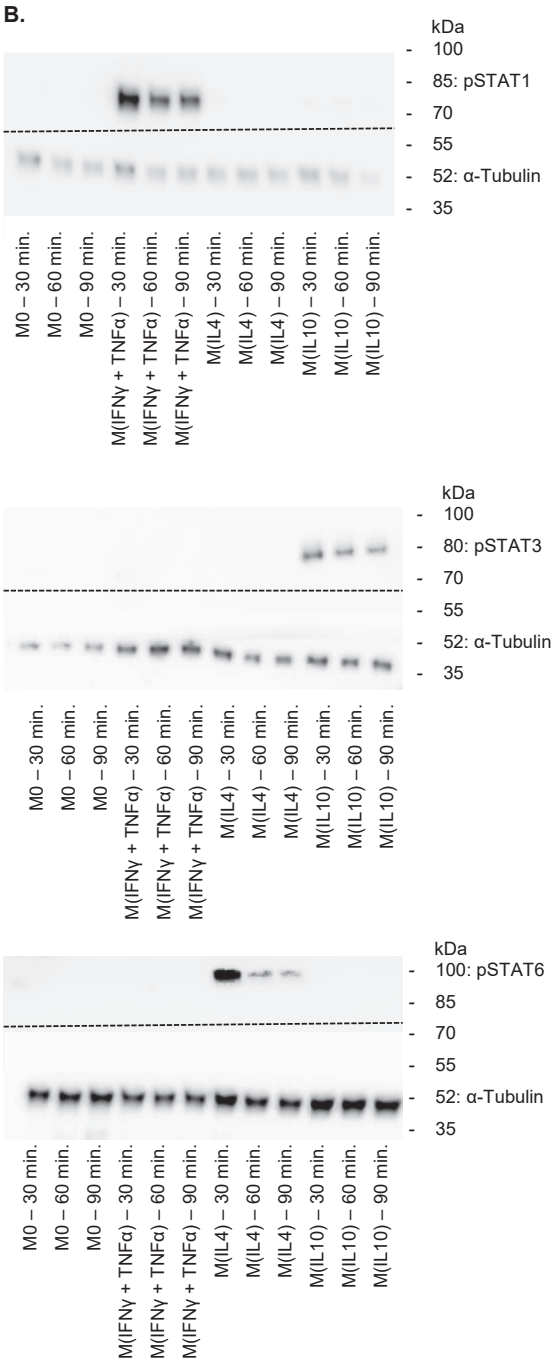
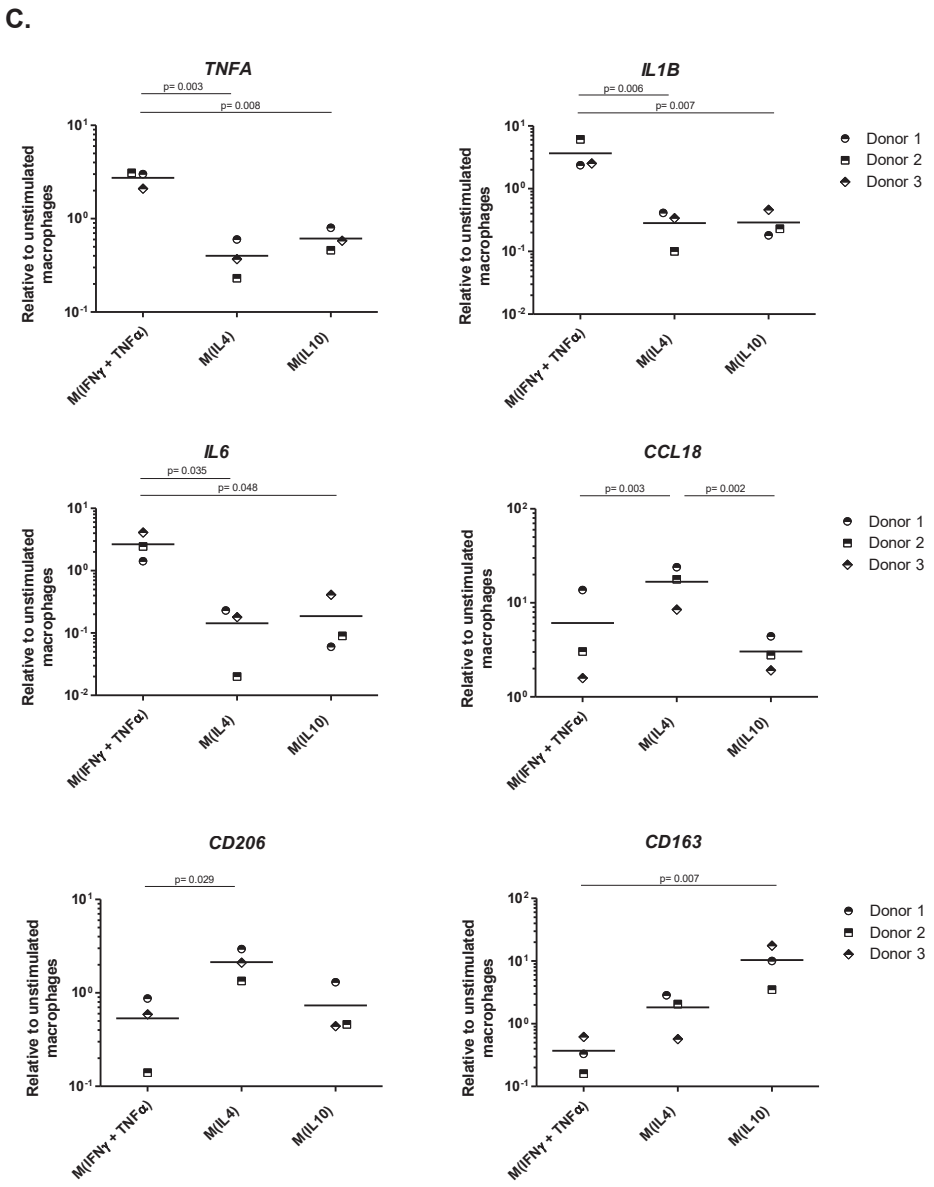


Figure 4. Differences in macrophage phenotypes in OA synovium and characterization of primary human macrophage phenotypes, stimulated with IFNγ and TNFα (M (IFNγ + TNFα)), IL4 (M(IL4)) or IL10 (M(IL10))



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D.

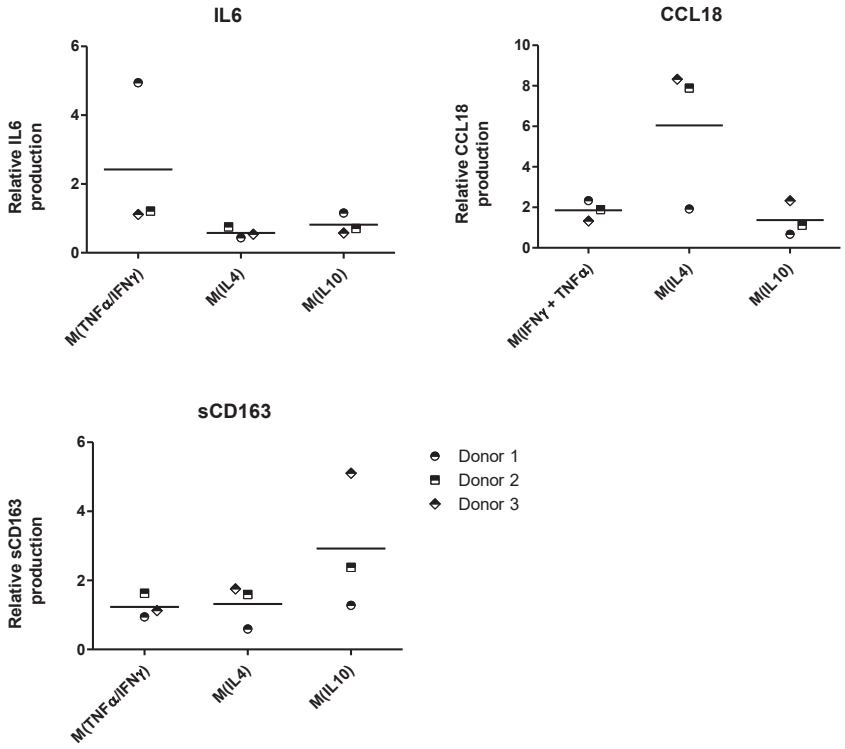

Figure 4. Differences in macrophage phenotypes in OA synovium and characterization of primary human macrophage phenotypes, stimulated with IFNγ and TNFα (M (IFNγ + TNFα)), IL4 (M(IL4)) or IL10 (M(IL10))

A) *Immunohistochemistry for markers indicating different macrophage phenotypes in OA synovium.* ***B)*** *Western Blot analysis of in vitro differentiated macrophages, at three time points after the start of differentiation.* ***C)*** *Gene expression corrected for GAPDH in the differentiated macrophages 24 hours after the start of stimulation, and D) Protein production by differentiated macrophages after 24 hours of IL6, CCL18 and sCD163 corrected for amount of DNA. Data is shown as mean (indicated by line) for n=3 donors analysed in 3-fold.*

DISCUSSION

In the current paper, we show that STAT1 and STAT3 phosphorylation levels differ among the OA synovial donors and that pSTAT6 is only detectable in presence of corresponding stimuli. Although NSC118-218 is the known STAT1 inhibitor, STAT1 phosphorylation was only inhibited by AS1517499 (known as STAT6 inhibitor) in presence of synovial fluid and resulted in a significant decrease of *IL1B* and *IL6* gene expression levels. In absence of synovial fluid, the STAT3 inhibitor S3I-201 inhibited STAT3 phosphorylation

and resulted in more *TNFA*, and less *IL6* and *CD163*. Since macrophage phenotypes had specific STAT1, 3, and 6 phosphorylation levels and presence of the macrophage phenotypes was confirmed in OA synovium, the targeting of phosphorylated STAT proteins within macrophage phenotypes might be a potential new approach to modulate synovial inflammation.

Nowadays, many anti-inflammatory compounds are being tested as potential new strategies for OA, focusing on complete suppression of inflammation, either via macrophages or via directly inhibiting cytokines. However, this approach may be too aspecific as sometimes a certain level of inflammation is required for a proper healing[20] and the composition of macrophage phenotypes in the synovium can differ at different stages of OA[3] and even between patients[12]. Many cytokines use JAK-STAT signaling pathways to transduce intracellular signals. As phosphorylated STAT proteins are found in the synovial membrane of patient with rheumatoid arthritis[9, 34, 36], modulation of intracellular signaling pathways have shown to be a promising intervention[2]. Compounds used in the current study share a comparable mechanism of action, as NSC-118218 and AS1517499 are known to inhibit tyrosine phosphorylation and S3I-201 binds to SH2 binding sites[6, 13, 21, 26]. This prevents STAT proteins to get phosphorylated and detached from its receptor. Ultimately resulting in an inability to form STAT dimers and therefore an inability to bind DNA recognition sequences. To our knowledge, this is the first study showing modulation of the inflammatory phenotype of osteoarthritic synovial tissue using STAT phosphorylation inhibitors.

Different culture set-ups were used when testing the effect of the inhibitors. Since pSTAT6 was only detectable when stimuli were present, we cultured synovial explants with synovial fluid while adding the three STAT-inhibitors. Here surprisingly enough only AS1517499, chosen as STAT6 inhibitor, decreased the phosphorylation of STAT1 but not STAT6. When the synovial explants were cultured in medium without synovial fluid, no STAT6 was detectable and thus no STAT6 inhibitor was tested. Without the presence of synovial fluid, NSC-118218 decreased STAT1 phosphorylation, but without changing expression of the analysed genes and S3I-201 decreased STAT3 phosphorylation in 4 of the 5 synovial explants donors in the presence of S3I-201. Upregulation of *TNFA* in response to inhibition of STAT3 phosphorylation may be explained by the action of different STATs: selective blockade of one STAT molecule might be compensated by more activation of another STAT molecule[7], although we did not see this for the analysed STATs. Another explanation for the increase of *TNFA* when STAT3 phosphorylation is inhibited might be that inhibition of anti-inflammatory markers abolishes a more pro-inflammatory response, as has been shown in previous work[30]. The loss of inhibitory function of NSC-118218 and S3I-201 in the presence of synovial fluid might be explained by a continuous presence of JAK-STAT pathway-activating stimuli in the synovial fluid[24], such as the STAT1 activators IL-1β and IL-6 or the STAT3 activator IL-10.

Our study showed that different macrophage phenotypes had specific STAT phosphorylation patterns *in vitro*. Presence of these corresponding macrophage phenotypes in OA synovium was confirmed and in line with previous reports[12, 28]. The levels of phosphorylated STAT proteins that could be detected in OA synovium appeared to be strongly donor dependent. This suggests that the stage of inflammation of the synovial tissue and possibly also the different macrophage phenotypes residing in the synovium varies among patients. Differences in the response to treatment with STAT-inhibitors might be explained by these findings.

A potential approach to modulate synovial inflammation might be via modulation of macrophages with high STAT1 phosphorylation levels, as these phosphorylated proteins are predominantly found in pro-inflammatory macrophages. On the other hand, one should avoid the modulation of macrophages with high pSTAT3 or pSTAT6 levels, since these phosphorylated STATs are associated with macrophages that have an anti-inflammatory phenotype. Therefore, modulating synovial inflammation by targeting phosphorylated STAT proteins in macrophages might be a suitable approach to delay the progression of OA.

To quantify the effect of the compounds on STAT phosphorylation we semi-quantitated the Western blot data. Besides, using total protein measurements to load an equal amount of protein per sample, we used α-tubulin as an extra control for normalization. We specifically chose to use α-tubulin for this purpose because we were interested in the total amount of pSTAT1, 3, and 6 irrespective of how much unphosphorylated STAT was present in the cell.Moreover, since phosphorylation of STATs might result in an altered ratio between STAT and pSTAT, unphosphorylated STATs cannot be used as control protein for equal loading.

In conclusion, different macrophage phenotypes have specific STAT phosphorylation levels. OA synovium contains these macrophage phenotypes but has varying STAT phosphorylation levels among the donors. This suggests that the composition of synovial macrophages and herewith the degree of synovial inflammation is strongly donor dependent. In addition, this study shows that inhibition of STAT phosphorylation in OA synovium modulates its inflammatory phenotype. Considering the varying STAT phosphorylation levels in OA synovium among patients, inhibition of STAT phosphorylation is a potential personalized therapeutic approach to direct the synovial inflammation seen in OA.

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CHAPTER 7

General discussion

The anterior cruciate ligament (ACL) is an important stabilizing ligament of the knee and commonly injured in pivoting sports. When surgical intervention is needed to restore knee stability, autogenous hamstring tendons are the graft of choice for ACL reconstructive purposes. The surgical harvesting of one or two hamstring tendons is required and subsequently used to reconstruct the ruptured ligament. In the light of potential donor site morbidity and functional deficits, both patients and orthopaedic surgeons voice concerns about the harvest of healthy and functional tendon tissue. However, in 1992 Cross et al. were the first to describe the potential of hamstring tendons to regenerate following harvesting procedures[21]. If regeneration takes place and regenerated tendons resemble the native ones, the post-harvest morbidity might be limited.
Therefore, the general aim of this thesis was to improve the outcome following harvest of the hamstring tendons through a better understanding of tendon regeneration. This might contribute to the identification of new (therapeutic) targets and ultimately result in an improved outcome after ACL reconstruction procedures entailing the hamstring tendons.

THE REMARKABLE CAPACITY OF HAMSTRING TENDONS TO REGENERATE

Despite the efforts to use synthetic materials or allografts, autografts remain the first choice for ACL reconstruction purposes[53, 56]. Today, the hamstring tendons are the most commonly used autograft to reconstruct the torn ACL[34, 56]. More specifically it requires the surgical resection of either the semitendinosus tendon only, or both the semitendinosus and gracilis tendon to prepare the graft. Another popular and widely used tendon autograft is the bone-patellar tendon-bone (BPTB) graft entailing the central third of the patellar tendon[34, 56]. Although the quadriceps tendon and iliotibial band are less frequently used as autografts, these are suitable alternatives too. Regardless the choice of autograft, surgical resection of healthy and functional tissue is needed. Both patients and surgeons raise concerns about the potential donor site morbidity and lack of this tendon for functional deficits.

What are the regeneration rates for hamstring tendons?

In 1992, Cross et al. were the first authors describing the remarkable regeneration capacity of the hamstring tendons after being entirely resected[21]. In our systematic review we showed that regeneration of the semitendinosus and gracilis tendons occurs in 70% within the first year following harvesting procedures (**Chapter 2**). Studies reporting about hamstring tendon regeneration used different imaging techniques, such as computed tomography (CT), magnetic resonance (MR) imaging and ultrasound.

In addition, various definitions were used to assess (in)complete regeneration of the hamstring tendons, regeneration was assessed dichotomously and evaluated only after a single follow-up period. This does not reflect a dynamic and continuous process such as regeneration of the hamstring tendons. Therefore, we described regeneration rates both one and two years after harvest using MR imaging (**Chapter 3**). In line with previous findings, we found that the semitendinosus tendons regenerated in 65.7% of the cases and that the gracilis tendons regenerated in 82.9% of the cases. Interestingly, regeneration rates in the second year after harvest were found to be lower compared to the regeneration rates one year after surgical resection. This might be explained by the observation that the initial structure is predominately fibrous with only a few collagen fibers[27]. Over time the regenerated tendon starts the remodeling process and becomes similar to the native tendon with longitudinally oriented collagen fibers that appear to be of appropriate orientation and dimension[33, 67, 80]. In line with these findings, biomechanical properties of regenerated tendons improve over time[54]. Therefore, regenerated structures might be most prone to rupture within the first period following harvest and result in a decline in the regeneration rates over time. Patients with a rupture of the regenerating structure often experience a sudden, persistent and sharp pain at the posterior thigh[63].

Hamstring tendons are not the only tendons that are frequently harvested for reconstructive purposes. Another often-used graft for ACL reconstruction procedures is the BPTB autograft, involving the harvest of the middle-third of the patellar tendon. Previous studies reported similar regeneration rates for the patellar tendon as aforementioned for the hamstring tendons[7, 88]. Another tendon that is harvested for ligamentous reconstruction tendon interposition of the carpometacarpal joint is the flexor carpi radialis tendon. Reported regeneration rates for this tendon reach up to 79% 4 years after surgery[5].

Regenerated hamstring tendons are thicker and longer compared to the native tendons

Tendons are important for the transmission of skeletal muscle forces to bone. Appropriate regeneration of the tendon is therefore important to withstand mechanical loads, resulting in an increase of cross-sectional areas (CSA)[38, 48]. In line with these findings, regenerated semitendinosus and gracilis tendons show a doubling of the CSA of compared to the CSA of the native tendons (**Chapter 3**). A similar increase of the CSA is observed after tendon resection for the BPTB autografts[4, 6, 7, 20, 45]. There are several feasible hypotheses that explain the increased CSA of regenerated tendons. First of all, the organization and composition of the extracellular matrix (ECM) in regenerated tendons might be inferior to the original tendon, causing diminished biomechanical properties. Therefore, more tissue is required to withstand the same mechanical forces as before. Secondly, the tendon is exposed to more mechanical stress per unit and therefore increases its CSA. A

third feasible explanation is that the increase in CSA serves as a protective mechanism to strengthen vulnerable tendons, as seen in Achilles tendinopathy and after Achilles tendon rupture[46, 74].

In line with the first hypothesis, it has been observed that in the first months after resection the regenerated structure is predominately fibrous with only a few collagen fibers[27]. Over time the regenerated tendon starts the remodeling process and becomes similar to the native tendon with longitudinally orientated collagen fibers[33, 67, 80]. Along with this improved organization of the ECM, the biomechanical properties of the newly formed structure ameliorate with the passage of time[54]. However, the ultimate load, stiffness and the modulus of the regenerated structure do not become identical compared to the native tendons. These inferior biomechanical properties may be partially due to the decreased cross-sectional diameter of the collagen fibers in the regenerated tendons[33]. This all fits within the first hypothesis and explains our observation that regenerated tendons have increased CSA compared to the native tendons.

The musculotendinous junctions (MTJ) of regenerated hamstring tendons appear to be found more proximal compared to the MTJ of native tendons, resulting in an increase of the semitendinosus and gracilis tendon length (**Chapter 3**). The average length of this proximal shift ranges from 3.1 to 7.3 cm[17, 62]. On the contrary, it is interesting to note that the patellar tendon is significantly shortened between 0.4 and 1.8 cm after harvest of the middle third of the tendon[7, 12].The opposite post-harvest remodeling of the patellar tendon and the hamstring tendon length might be explained by the different anatomic situations after surgical intervention. The hamstring tendons are harvested from insertion to the MTJ, leaving a free anatomic space between the fascial planes of the medial thigh. However, a BPTB graft requires a longitudinal incision directly over the patellar tendon, involving harvest of the middle third and leaving two thirds of the native tendon in situ. In addition, some surgeons close the harvest gap. This surgical approach might result in formation of exaggerated pathologic fibrous hyperplasia causing a shortening of the patellar tendon and a subsequent tendon shortening[69].

Recently, Laako et al. described a surgical technique in which the distal head of the harvested semitendinosus and/or gracilis muscle is drawn towards its anatomical location and attached to the semimembranosus muscle[50]. This technique could be of value in patients with increased lengths of the semitendinosus and gracilis tendons, since increased tendon lengths might cause symptoms such as posterior thigh pain. Alternatively, one could choose to only harvest 75% of the tendons, leaving the other 25% *in situ*. Another, relatively rare, symptom caused by the altered thickness and length of regenerated tendons is a snapping syndrome. Gali et al. proposed a method of percutaneous lengthening of regenerated tendons alleviating symptoms and resulting in minimal morbidity[30].

The work in this thesis emphasizes that tendons regenerate and that regenerated hamstring tendons are thicker and longer than the native ones, altering the anatomy and biomechanics. Therefore, patients may experience symptoms that are related to this new situation. Orthopaedic surgeons should be aware of the potential of the semitendinosus and gracilis tendons to regenerate, their altered morphological properties and the potential accompanying clinical symptoms. If needed, surgical interventions should be considered.

Should hamstring tendons be reconsidered as graft of choice for ACL reconstruction purposes?

Patients often express their concerns regarding potential muscle strength deficits following tendon harvesting procedures. Previous studies measuring the peak torque of knee flexion reported a full recovery of hamstring strength[19, 55, 87]. This recovery might be attributed to a functional restoration of muscle-tendon-bone complex of the hamstring tendons. Alternatively, it might also be caused by compensatory hypertrophy of the remaining knee flexors, such as the biceps femoris and semimembranosus[26, 42]. Since the semitendinosus and gracilis muscles insert at the pes anserinus and are therefore more important for higher angles of knee flexion (beyond 75 degrees)[65], strength deficits might be found in deep knee flexion. In line with this hypothesis, three studies reported strength deficits ranging from 20 to 30% at knee flexion angles beyond 75 degrees[17, 64, 82]. However, the clinical relevance of this limited strength deficit is debatable (**Chapter 4**).

Orthopaedic surgeons worldwide have not reached consensus yet regarding the selection of the best graft to reconstruct ruptured ACLs. Based on several studies, hamstring tendons and bone-patellar tendon-bone are the most frequently used autograft[1, 34, 56]. However, BPTB autografts result in significantly more donor site morbidity and lower patient reported outcome measurements and hence hamstring tendons are the graft of choice for ACL reconstructions[1, 34, 56, 57]. However, the semitendinosus and gracilis tendons are primarily internal rotators of the tibia, withstanding excessive external rotation and protecting the (reconstructed) ACL[3, 68, 75]. Patients without hamstring tendon regeneration have impaired internal tibial rotary strength[2, 3] and might therefore be at increased risk for ACL re-ruptures[3]. Preoperative identification of patients that are likely to lack regenerative capacity of the hamstring tendons might alter the graft choice. Since there is currently no literature available that supports this hypothesis, future studies should investigate if regeneration indeed decreases the risk of ACL re-rupture.

Another interesting aspect of regenerated hamstring tendons is their potential to be re-harvested. Although it has been reported that the semitendinosus and gracilis tendons have been reharvested for ACL[89] and medial patella-femoral ligament (MPFL)[80] reconstructions, the re-use of these tendons is still very questionable for several reasons. First of all, tendon regeneration does not occur in every patient and therefore harvesting

is not always possible. Given the morphological features of the remodeled tendons and the importance of graft thickness and graft length, newly formed tendons could be considered as potential interesting candidates for reharvesting procedures. However, it is important to address that the strength and stiffness of the newly formed tendons is expected to be inferior to the native tendons. In addition, histological studies revealed that the remodeled tendons show areas of scar tissue that could be expected to alter graft strength[25, 27, 67].

Taken together regenerated tendons resemble the native tendons, but are not identical in terms of morphology, histology and biomechanical features. It would be helpful to identify key role players of tendon regeneration and remodeling because it contributes to the identification of targets to direct and improve these processes. Ultimately, this knowledge might be used to improve treatments for tendon-related diseases.

MODULATORS FOR THE INFLAMMATORY RESPONSE

Tendon repair is a complex and multistage process, which is initiated after tissue damage and ultimately results in function restoration. Although numerous cells are involved in the process of tendon repair, macrophages are thought to be key role players in this process[35, 49].Various *in vitro* studies showed that the different macrophage phenotypes produce distinct factors, having different effects on tendon repair. Considering the specific effects of the specific macrophage phenotypes, it is not surprising that macrophage subsets are differently recruited during the process of tendon repair[58, 73]. More specifically, it has been shown that M1-like macrophage are recruited first and stimulate tenocytes to produce catabolic enzymes, such as matrix metalloproteinases[73]. In addition, M1-like macrophages typically produce factors such as IL-1β, IL-6, and TNF- α[84]. In **Chapter 5**, we describe that pro-inflammatory cytokines negatively influence the production of structural and fibril-associated ECM components. Then, M2-like macrophages are recruited resulting in higher ECM densities and increased collagen I expression presenting an orientation along the longitudinal axis of the tendon[58]. This macroscopically resembles a normal and healthy tendon. Together, these findings emphasize the pivotal role of macrophages in the tendon repair process. However, it is important to note that both pro-inflammatory and anti-inflammatory macrophages/cytokines are highly required for optimal tissue healing: inhibition of pro-inflammatory factors and stimulation of anti-inflammatory factors will not necessarily result in proper tissue healing. Tissue healing strongly depends on the finely regulated balance between pro- and anti-inflammatory factors. The question remains whether aging and smoking that are known to negatively influence regeneration chances (**Chapter 4**) also affect macrophages and/or their phenotypes.

Aging

Aging is associated with decreased chances for regeneration of the hamstring tendons (**Chapter 4).** In general, it is known that aging negatively affects the function of the immune system[61].

Currently, it is unclear whether the generation of macrophages from monocytes is impaired with age[39, 66, 85]. Regarding macrophage polarization, it has been reported that more M2-like macrophages are found in a mouse-model of age-related macular degeneration[47]. These ocular macrophages had decreased levels of TNF-α and IL-12, whereas the anti-inflammatory cytokine IL-10 was upregulated. In addition, an increase in M2-like macrophages was observed with aging in spleen, lymph nodes and bone marrow[43].

Aging influences the cytokine secretion patterns by macrophages. Compared to cytokine production in young mice, peritoneal macrophages from old mice secrete less pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF- α, and more anti-inflammatory cytokines such as IL-10[9, 14, 72]. Microarray analysis further investigated the molecular basis for this decrease and revealed signal transduction genes were specifically reduced in macrophages from old mice[15]. First of all, decreased expression of toll-like receptor (TLR) 4 has been suggested as a reason for the observed age-related alterations[72]. However, other studies indicated that TLR4 remains unchanged with age[9]. Another explanation might be alterations in the JAK-STAT signaling pathway. As shown in **Chapter 6**, macrophage phenotypes have a characteristic STAT phosphorylation pattern. Previous studies showed that STAT1 phosphorylation levels are decreased in macrophages from old mice, compared to young mice[23]. Since STAT1 phosphorylation is mainly found in M1-like macrophages, decreased phosphorylation levels might therefore contribute to reduced production of pro-inflammatory cytokines.

Taken together, aging impacts on macrophage function and potentially disturbs the highly regulated balance between pro- and anti-inflammatory factors, which is required for optimal tendon healing. A failure to repair the ECM of tendons might therefore be caused by an impaired macrophage function.

Smoking

Smoking has been shown to have deleterious consequences for several orthopaedic conditions, such as higher rates of hip fracture, nonunion of fractures and osteomyelitis. In addition, smoking is also associated with impaired regeneration of the hamstring tendons (**Chapter 4**).

M1-like macrophages play a central role in defending the human body against invading pathogens and foreign material. Cigarette smoking reduces the phagocytic ability of macrophages[40] and decreases levels of molecules that are needed for intracellular killing, such as nitric oxide (NO) and reactive oxygen species (ROS)[90]. This finding suggests that

cigarette smoke induces macrophage polarization towards a M2-like phenotype. This hypothesis is further strengthened by the activation of STAT3 pathways in macrophages after exposure to cigarette smoke[32]. Activation of this pathway is seen in M2-like macrophages (**Chapter 6**) and needed for the production of anti-inflammatory cytokines. In addition, low levels of cytokines that are typically produced by M1-like macrophages are found in response to cigarette smoke[16]. Therefore, STAT3 is considered to be a pivotal signaling molecule for macrophage polarization towards an M2-like phenotype.
Similar to the effect of aging, the exposure to cigarette smoke influences the tightly regulated equilibrium between pro- and anti-inflammatory factors and herewith potentially affects the repair capacity of the ECM.

Genetics

The aforementioned factors only partially explain the interindividual variation in tendon repair. Today, there is mounting evidence suggesting that genetics has a pivotal role in the healing tendency of tendons[70, 76].
Tendons are subject to mechanical loads that reach up to ten times an individual's body mass. Therefore, the ECM of tendons continuously needs to undergo remodeling in order to withstand these loads and maintain homeostasis. It has been described that tendons are able to respond to these mechanical loads by initiating several matrix remodeling pathways[52, 86]. Previous studies showed that inflammatory gene expression profiles of tenocytes are modulated in response to mechanical loading triggering tenocyte apoptosis and ECM degradation. Therefore, it is reasonable to hypothesize that polymorphisms within genes encoding for inflammatory proteins modulate risk of tendinopathy.
Previous studies showed that genes encoding several interleukin proteins including interleukin-1β (*IL1B* rs16944 C>T), interleukin-6 (*IL6* rs1800795 G>C) and interleukin-6 receptor (*IL6R* rs2228145 A>C) modulate risk of Achilles tendinopathy in cohorts from South Africa[77], Australia[77] and the United Kingdom[10]. All these polymorphisms are located at the promotor site, affecting the produced amounts of the respective interleukines[12, 28, 31, 51].
It has been shown that *IL1B* rs16944 is independently associated with an increased risk of acute Achilles tendon ruptures[10]. For chronic Achilles tendinopathy, no independent associations were noted[77]. However, *IL1B* and *IL6* variants were associated with increased risk of Achilles tendinopathy in inferred gene-gene interactions models[77]. Although it has been well described that IL-1β and IL-6 are responsible in the activation of many downstream signaling cascades, it remained unclear how these polymorphisms contribute to tendinopathy[70, 76]. The work in this thesis indicated that polymorphisms within genes encoding IL-6 and IL-1β modulate the production of structural and fibril-associated ECM components (**chapter 5**) and herewith potentially contribute to an impaired capacity to repair the ECM.

It is interesting to note that additional associations were found between polymorphisms within genes encoding for *IL1B* and *IL6*, and other soft musculoskeletal injuries. In line with the findings for acute rupture of the Achilles tendon, the *IL1B* rs16944 promotor polymorphism independently increases the risk of ACL rupture[71]. In addition, inferred allele combinations of *IL1B*, *IL6* and *IL6R* were found to be associated with risk of ACL ruptures, both two independent South African cohorts[71] and a Swedish cohort (**chapter 5**). The risk of developing carpal tunnel syndrome has also shown to be modulated by polymorphisms within IL6R[11]. Collectively, these results underline the potential implication of interleukin signaling pathways in the underlying mechanism that predisposes for both acute and chronic soft musculoskeletal injuries. The investigated polymorphisms were found to be associated with increased risk for ACL rupture in a Swedish cohort (**Chapter 5**), and other cohorts[71]. In addition, these polymorphisms were previously associated with increased risk of chronic Achilles tendinopathy[70, 76, 77]. Therefore, it might be suggested that these polymorphisms negatively affect the odds for hamstring tendon regeneration.

Personalized medicine is a central dogma in current clinical practice, proposing the tailor made clinical assessment of an individual patient based on their extrinsic and intrinsic risk factors[18]. Tendon healing is based on a poorly understood complex interaction between a variety of extrinsic and intrinsic risk factors. Clinicians should be aware of this when considering any clinical application of genetic testing and prevent the use of terms such as diagnostic, prognostic or preventive. Instead, clinicians should rather consider injury susceptibility through the identification of both known intrinsic and extrinsic risk factors (Figure 1). Individuals who are at increased risk to develop a soft musculoskeletal injury should then be referred to a sports-trained physiotherapist to be managed personally by appropriate prehabilitation exercises to reduce risk.

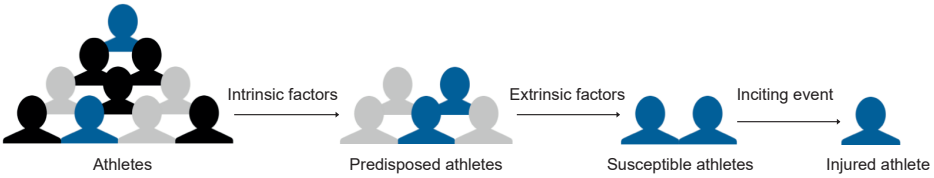

Figure 1: Schematic overview of the complex relationship between intrinsic and extrinsic factors, as well as the role of the inciting event. Future multifactorial models could be of great value to distinguish low-risk (black), predisposed (grey) and susceptible (blue) athletes.

FUTURE PERSPECTIVES

Remaining uncertainties about tendon regeneration

The work in the current thesis shows that the semitendinosus and gracilis tendons regenerate in 70% of patients within the first two years following tendon harvest for reconstructive purposes. It appears that these tendons regenerate in a proximal to distal fashion, and that regenerated tendons are longer and have increased cross-sectional areas compared to the native tendons. However, the exact mechanism that underpins hamstring tendon regeneration remains unclear. In the field of Developmental Biology and Cancer, lineage tracing is currently the golden standard to determine a cell's origin. In this technique, a single cell is labeled in such a way that the mark is transmitted to the cell's progeny. The advantage of this approach is that it can be performed without any prior knowledge of what genes or markers should be expressed. However, a disadvantage this technique requires to physically stop the development process to see how cells look. Therefore, more recent developments have enabled *in vivo* barcode generation, targeting a locus for rearrangement or mutagenesis such that a different set of outcomes is generated in different cells[44]. The barcodes are generated over a limited amount of time resulting in a deep and precise lineage tracing. A great advantage of this strategy is the ability to continuously record a cell's development. Another more indirect method to identify the origin of the cells residing in the newly formed tendon tissue is to compare its methylome with the methylomes of cells derived from the surrounding tissues, such as fat, muscle and tendon sheets. A methylome is the methylation of cytosines, contributing to the epigenetic layer and defining the transcriptional and regulatory potential of genomic DNA[41].

Mid- and long-term follow-up of patients with harvested hamstring tendons is necessary to further evaluate the clinical implications of tendon regeneration. The semitendinosus and gracilis tendons withstand excessive external tibial rotation protecting the ACL. An impaired repair capacity of the hamstring tendons following harvesting procedures might therefore impact ACL reconstruction survival rates. Although there is currently no literature available on this topic, hamstring tendon regeneration is potentially of importance for the clinical outcome after ACL reconstruction procedures. Additionally, complete hamstring tendon regeneration theoretically re-establishes a functional muscle-tendon-bone complex. This hypothesis is supported by the current literature, suggesting that tendon regeneration would result in no or limited loss of muscle strength. However, it remains unclear whether this is caused by high-quality regeneration or compensatory hypertrophy of other (posterior) thigh muscles. In this light, it would be highly valuable to evaluate the volumes of all thigh muscles following harvesting procedures. Next to this, the quality of the regenerated tendons might be indirectly measured by the radiologic appearance of the hamstring muscles. Previously tendon-related disorders of the rotator

cuff have been described to cause a myriad of changes in the cuff musculature on MR imaging, such as fatty infiltration, atrophy and fibrosis. In line with these observations, one might hypothesize that impaired regeneration of the hamstring tendons causes similar deviations of the semitendinosus and gracilis muscles.

Improved understanding of tendon- and ligament related injuries

ACL injuries are often associated with knee instability, take up to 9 months to rehabilitate and lead to gonarthrosis in 40% of the patients 10 years after injury[59]. In addition, treatment of ACL injuries is costly and only 50% of the athletes successfully return to preinjury levels[29, 59]. Therefore, the current focus of musculoskeletal research is the identification of factors associated with increased susceptibility to ACL injuries. Independent associated factors with ACL injury include anatomical variations, neuromuscular control, sex, female sex hormone concentrations, genetic polymorphisms and previous injuries[78, 79]. To date, studies that evaluated risk of ACL injury using combinations of risk factors by developing multivariable models are limited[36, 83]. These models exclusively focus on the anatomic features and do not provide a full understanding of ACL injury risk. However, it is probable that tendon- and ligament related injuries depend on multiple risk factors. Therefore, it is important to focus on creating comprehensive and clinically applicable risk models identifying individuals with increased susceptibility to both tendon- and ligament-related injury. Besides, the models should provide more direction for preventive programs, as well as appropriate counseling for those who are at increased risk.

The healing process of tendons involves an inflammatory phase. Macrophages are considered to be pivotal in the onset and perpetuation of tendon diseases[22, 60, 81]. Some studies indicate that macrophage depletion improved morphological and biomechanical properties in injured Achilles tendons or following ACL reconstruction procedures[24, 37]. On the other hand, other studies reported that aspecific inhibition of macrophages is detrimental to ECM formation and tensile strength[8, 13]. In line with these findings, aspecific targeting of inflammation by common anti-inflammatory drugs including diclofenac, celecoxib and naproxen seem to have deleterious effects on tendon healing. These findings emphasize the functional role of macrophages in the tendon-healing process but suggest the need for further clarification on the interplay between macrophages and tenocytes. *In vitro* studies are limited but suggest that different macrophages have different effects on tenocyte behavior[58, 73, 81]. Given these different effects of the different macrophage subsets, it might be interesting to specifically target macrophages in order to improve the tendon remodeling process. This can either be done by using specific antibodies to specifically target a monocyte or macrophage subset, or by modulating specific intracellular signaling proteins, such as STAT proteins.

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CHAPTER 8

Summary

When surgical intervention is needed to reconstruct a ruptured anterior cruciate ligament (ACL), the hamstring tendons are often harvested and subsequently used as autograft. The harvested hamstring tendons have the potential to regenerate. The main of this thesis was therefore to improve the clinical outcome following harvest of the hamstring tendons through a better understanding of the process of hamstring tendon regeneration.

Chapter 2 summarized the available literature on hamstring tendon regeneration and showed that 70% of the patients have the potential to regenerate their harvested tendons. However, the included studies assessed hamstring tendon regeneration dichotomously, used multiple definitions for tendon regeneration and only evaluated regeneration at a single follow-up period. Therefore, **Chapter 3** evaluated hamstring tendon regeneration rates both 1 and 2 years after harvest using magnetic resonance (MR) imaging. We found that the semitendinosus tendons regenerated in 65.7% and that the gracilis tendons regenerated in 82.9% of the cases. In addition, regenerated hamstring tendons were found to have significantly increased cross-sectional areas and lengths compared to the original tendons. Failure to regenerate and the altered morphological properties might cause clinical symptoms such as posterior thigh pain, cramping and weakness. In this light, it might be interesting to preoperatively identify individuals with poor chances of complete regeneration of the hamstring tendons. **Chapter 4** revealed that aging and smoking were negatively associated with regeneration chances. In addition, it revealed that patients without regeneration reported higher pain scores compared to those with regenerated tendons.

Another well-known factor involved in tissue repair processes is inflammation. **Chapter 5** revealed that polymorphisms within genes encoding inflammatory proteins such as *IL6* and *IL1B* affect the production of structural and fibril-associated extracellular matrix components in a risk-dependent manner. In addition, it was found that these polymorphisms contribute to risk of ACL injuries. Taken this together, these results suggest that *IL6* and *IL1B* might be important factors during the process of hamstring tendon regeneration. In order to direct the inflammatory process, **Chapter 6** focused on the modulation of inflammation using activated STAT-signaling pathways in macrophages. Specific inhibition of activated STAT proteins modulated the inflammatory phenotype, potentially via modulating macrophage phenotypes.

CONCLUDING REMARKS

To conclude, the work in this thesis described the regenerative capacity of hamstring tendons following harvesting procedures. In the future, clinicians should preoperatively inform patients about tendon regeneration, its clinical consequences and possibly alter the choice of graft. Furthermore, this thesis identified potential targets to improve tendon

regeneration. Future studies on the role of inflammation and tendon repair could focus on the role and impact of macrophages.

APPENDICES

Nederlandse samenvatting

List of publications

Ph.D. portfolio

Curriculum vitae

NEDERLANDSE SAMENVATTING

De voorste kruisband speelt een essentiële rol bij de stabilisatie van het kniegewricht. Een ruptuur van de voorste kruisband is een van de meest voorkomende sport-gerelateerde letsels van de knie, waarbij in de helft van de patiënten een hersteloperatie geïndiceerd is. Tijdens een operatie worden veelal twee hamstringpezen, de semitendinosus en gracilis pees, gebruikt om de gescheurde kruisband te reconstrueren. Deze pezen worden chirurgisch verwijderd vanaf de spier-pees overgang tot op de aanhechting aan het onderbeen. Opvallend genoeg blijken sommige patiënten in staat om deze geoogste pezen te regenereren. Het doel van dit proefschrift is derhalve ook om de klinische uitkomst na het oogsten van hamstringpezen te verbeteren door het proces van hamstringpees-regeneratie beter te begrijpen.

In **Hoofdstuk 2** werd de beschikbare literatuur met betrekking tot hamstringpees-regeneratie samengevat. Hierin werd aangetoond dat 70% van de patiënten beschikt over de capaciteit om de hamstringpezen te regenereren. Echter, het werd ook duidelijk dat in de huidige literatuur een eenduidige definitie van hamstringpees-regeneratie ontbreekt. Daarnaast werden verschillende technieken gebruikt om regeneratie in beeld te brengen en werd de regeneratiestatus altijd louter op één moment bepaald.

Derhalve werd in **Hoofdstuk 3** met behulp van MRI beelden hamstringpees-regeneratie geëvalueerd op zowel 1 als 2 jaar na het chirurgisch verwijderen van de oorspronkelijke pezen. Hieruit bleek dat de semitendinosus in 65,8% en de gracilis in 82,9% van de patiënten geregenereerd is in het tweede jaar na chirurgie. In vergelijking met het eerste jaar had 10,5% van de patiënten een veranderde regeneratiestatus, waarbij zowel verbetering als verslechtering van het regeneratieproces werd gezien. Daarnaast werd beschreven dat geregenereerde pezen een toegenomen oppervlakte en lengte hebben in vergelijking met de originele pezen. Zowel het falen van het regeneratieproces, als de veranderde morfologische peeseigenschappen kunnen leiden tot symptomen als spierkramp, pijn en zwakte in de achterzijde van het bovenbeen. Het is daarom ook klinisch relevant om voor de operatie de patiënten te identificeren die mogelijk een verminderde kans hebben op succesvolle regeneratie, zodat de operatieve behandeling eventueel kan worden aangepast. **Hoofdstuk 4** liet zien dat oudere mensen en rokers een verminderde kans hebben op regeneratie van de pezen. Bovendien werd duidelijk dat patiënten zonder regeneratie hogere pijnscores rapporteerden dan patiënten met geregenereerde pezen in het tweede jaar na de operatie.

Een andere factor waarvan bekend is dat die een rol speelt bij het herstel van weefsels is ontsteking. In **Hoofdstuk 5** werd duidelijk dat varianten in genen die coderen voor ontstekings-gerelateerde eiwitten, zoals *interleukine (IL) 1B* and *IL6* de productie van extracellulaire matrix componenten beïnvloeden. Daarnaast werd duidelijk dat deze genetische varianten het risico op het scheuren van de voorste kruisband beïnvloeden.

Het is daarom aannemelijk dat *IL1B en IL6* belangrijke factoren zijn voor het proces van hamstringpees-regeneratie. Om het regeneratieproces te beïnvloeden werd in **Hoofdstuk 6** onderzocht of het mogelijk is het ontstekingsproces te moduleren door het remmen van specifieke processen in macrofagen, ontstekingscellen die een rol spelen in weefselherstel. Door specifieke STAT-eiwitten te remmen konden we de eigenschappen van het ontstekingsproces beïnvloeden, mogelijk via macrofagen.
Concluderend heb ik in dit proefschrift de regeneratieve capaciteit van hamstringpezen beschreven. Dit maakt het in de toekomst mogelijk dat artsen patiënten voor de operatie beter informeren over hamstringpees-regeneratie, de klinische gevolgen hiervan en de operatietechniek hierop aanpassen. Bovendien laat het werk in dit proefschrift nieuwe potentiële aangrijpingspunten zien om peesregeneratie te beïnvloeden. Nieuw wetenschappelijk onderzoek naar de rol van ontsteking en peesherstel zou zich kunnen concentreren op de interactie tussen peescellen en macrofagen.

LIST OF PUBLICATIONS

High fat diet accelerates cartilage repair in DBA/1 mice
Wei W, Bastiaansten-Jenniskens YM, **Suijkerbuijk MAM**, Kops N, Bos PK, Verhaar JAN, Zuurmond AM, Dell'Accio F, Van Osch GJVM
Manuscript accepted. J Orthop Res 2017

Hamstring tendon regeneration after harvesting: a systematic review
Suijkerbuijk MAM, Reijman M, Lodewijks SM, Punt J, Meuffels DE
Manuscript accepted. AJSM 2015

Remodeling of regenerated hamstring tendons: a magnetic resonance imaging study
Suijkerbuijk MAM, Reijman M, Oei EHG, Van Meer BL, Van Arkel ERA, Meuffels DE
Manuscript submitted.

Predictive factors of hamstirng tendon regeneration and functional recovery after harvesting: a prospective follow-up study
Suijkerbuijk MAM, Reijman M, Oei EHG, Van Meer BL, Van Arkel ERA, Meuffels DE
Manuscript accepted. AJSM 2018

Functional polymorphisms within the inflammatory pathway regulate expression of extracellular matrix components in a genetic risk dependent model for anterior cruciate ligament injuries
Suijkerbuijk MAM, Ponzetti M, Rahim M, Posthumus M, Häger CK, Stattin E, Nilsson KG, Teti AM, Meuffels DE, Van der Eerden BJC, Collins M, September AV
Manuscript accepted. JSMS 2019.

Inhibiting phosphorylation of STAT proteins modulates the inflammatory phenotype of osteoarthritic synovium
Suijkerbuijk MAM, Çapar S, Kops N, Van Osch GJVM, Bastiaansen-Jenniskens YM
Manuscription in preparation.

Predictive factors for hamstring autograft diameter in anterior cruciate ligament reconstruction
Heijboer WMP, **Suijkerbuijk MAM**, Van Meer BL, Bakker EWP, Meuffels DE
Manuscript accepted. Knee Surgery 2019

Investigation of three independent populations strenghtens the hypothesis that genetic loci within the proteoglycan and angiogenesis associated pathways predispose to anterior cruciate ligament injury.
Feldmann D, Rahim M, **Suijkerbuijk MAM**, Cieszczyk P, Ficek K, Huminska-Lisowska K, Häger CK, Stattin E, Nilsson KG, Posthumus M, Collins M, September AV
Manuscript in preparation.

DANKWOORD

'*If I have seen further, it is by standing on the shoulder of Giants*'
Isaac Newton (1643-1727)

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PH.D. PORTFOLIO

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Co-promotors	Duncan E. Meuffels, MD, Ph.D.
	Yvonne M. Bastiaansen-Jenniskens, Ph.D.

PhD training

	Year	Workload (ECTS)
Courses		
Research integrity (EMC)	2018	0.3
Conferences – podium presentations		
Literature overview of hamstring tendon regeneration *EFORT meeting, Rotterdam, The Netherlands*	2015	1.0
Regenerated hamstring tendons after anterior cruciate ligament reconstruction on magnetic resonance imaging: a prospective observational follow-up study *17th ESSKA Congress, Barcelona, Spain*	2016	1.0
Inhibiting STAT-signaling pathways in macrophages to improve tissue regeneration *25th annual NBTE meeting, Lunteren, The Netherlands*	2017	1.0
Hamstringpees regeneratie: voorspellers en functionaliteit *NVA 2017 meeting, Rotterdam, The Netherlands*	2017	1.0
Predictors and functional recovery of hamstring tendon regeneration: a prospective observational follow-up study *11th Biennial ISAKOS congress, Shanghai, China*	2017	1.0
Inhibiting STAT signaling pathways in macrophages to improve tissue regeneration *TERMIS-EU 2017 conference, Davos, Switzerland*	2017	1.0
Polymorphisms within genes enconding inflammatory proteins modulate susceptibility for anterior cruciate ligament injury *Annual RUBICON meeting 2018, Manchester, United Kingdom*	2018	1.0
Functional polymorphisms within the inflammatory pathway regulate expression of extracellular matrix components in a genetic risk dependent model for anterior cruciate ligament injury *European College of Sports Science, 2019, Prague, Czech Republic*	2019	1.0
Investigation of three independent population strengthens the hypothesis that genetic loci within the proteoglycan and angiogenesis-assocatiated pathways predispose to anterior cruciate ligament injury *European College of Sports Science, 2019, Prague, Czech Republic*	2019	1.0
Conferences – poster presentations		
High fat diet accelerates cartilage repair in DBA/1 mice *19th Molecular Medicine Day, Rotterdam, The Netherlands*	2015	1.0

Hamstring tendon regeneration after harvesting: a systematic review *NVA 2015 meeting, Burgh-Haamstede, The Netherlands*	2015	1.0
Hamstring tendon regeneration after harvesting: a systematic review *10th Biennial ISAKOS Congress 2015, Lyon, France*	2015	1.0
Diminishing synovial inflammation by inhibition of STAT signalling *OARSI World Congress, Las Vegas, United States*	2017	1.0
Conferences		
Sporthopaedie; soccer doc *Rotterdam, The Netherlands*	2017	1.0
International scientific tendinopathy symposium *Groningen, The Netherlands*	2018	1.0
Annual RUBICON meeting *Manchester, United Kingdom*	2018	1.0
Department presentations and meetings		
Lab research discussion meetings	2015-2017	1.0
Journal Club *Department of Orthopaedics*	2015-2017	0.5
Lab-Clinics meeting *Department of Orthopaedics*	2015 - 2018	0.5
Orthopaedic Department Science day (anually)	2015, 2018	0.2
Miscellaneous		
Visiting PhD student at Department of Human Biology, Division of Exercise Science and Sports Medicine (6 months) *University of Cape Town, Cape Town, South Africa*	2018	5.0
Grants		
Erasmus Trustfonds, conference travel grant	2016	
Erasmus Trustfonds, conference travel grant	2017	

CURRICULUM VITAE

Mathijs Adrianus Maria Suijkerbuijk was born on October 14th, 1992 in Hoeven. In 2011 he obtained his gymnasium diploma at the Katholieke Scholengemeenschap Etten-Leur. After a local selection procedure (Decentrale Selectie), he started his medical training at the Erasmus MC, University Medical Center Rotterdam in September 2011.

Mathijs combined his medical training with the Research Master Molecular Medicine organized by the Erasmus MC. During a period of 12 months, he participated in a special Orthopaedic training program (Klinisch Excellentie Traject) organized by three teaching hospitals (Reinier de Graaf Ziekenhuis, Delft; Elisabeth-TweeSteden Ziekenhuis, Tilburg; and Erasmus MC, Rotterdam). After successful completion, he obtained his degree of Medical Doctor in November 2017.

In January 2015, he started the research at the department of Orthopaedic Surgery under supervision of Prof. dr. G.J.V.M. van Osch, dr. D.E. Meuffels and dr. Y.M. Bastiaansen-Jenniskens. As part of his PhD, he conducted research at the University of Cape Town, South Africa for a 6 months period under supervision of Prof. dr. M. Collins and Prof dr. A.V. September. This secondment was financially supported by a European Union's Horizon 2020 research and innovation program under a Marie Skłodowska-Curie grant.

From January 2019, Mathijs works as a resident (AIOS) at the Department of General Surgery at the Amphia Ziekenhuis in Breda (supervisor: Prof. dr. L. van der Laan). Then, he will continue his residency at the Department of Orthopaedic Surgery in the St. Elisabeth-TweeSteden Ziekenhuis (supervisor: Dr. T. Gosens) and the Erasmus MC (supervisor: Dr. P.K. Bos)