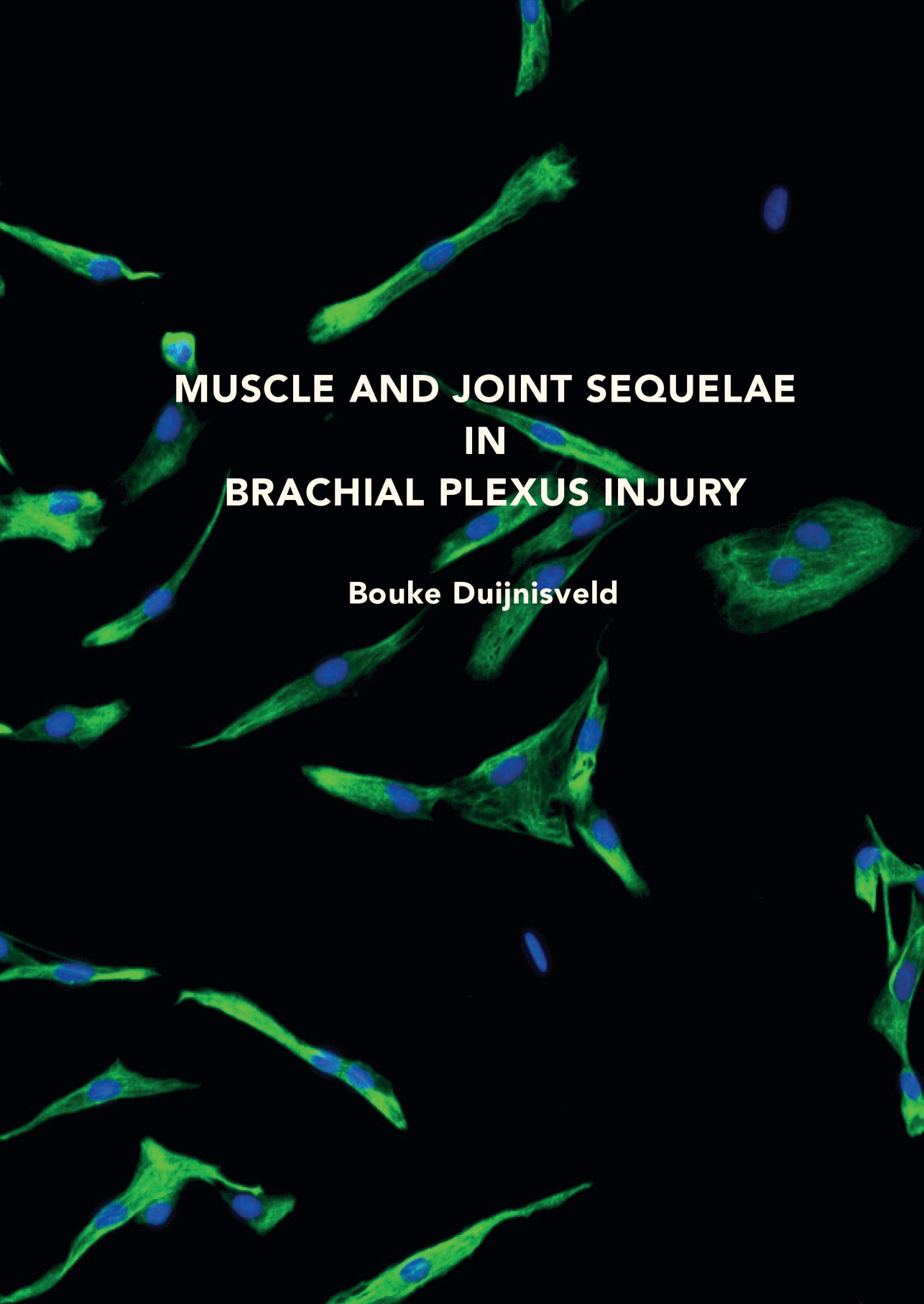

MUSCLE AND JOINT SEQUELAE IN BRACHIAL PLEXUS INJURY

Bouke Duijnisveld

Muscle and Joint Sequelae
in
Brachial Plexus Injury

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Aan mijn ouders

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Introduction

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INTRODUCTION

Brachial plexus injury

A brachial plexus injury (BPI) is caused by traction on the brachial plexus during delivery or due to a high-energy road traffic accident in young adults [1, 2]. Multiple synonyms are used for an obstetric brachial plexus injury (OBPI), including neonatal, congenital and perinatal brachial plexus palsy. However OBPI is the most preferred terminology in Europe and is therefore used in this thesis [3]. The incidence of an OBPI during delivery varies between 0.4 and 2.9 per 1000 live births [4-6]. The upper part of the brachial plexus (C5,C6) is most often affected, resulting in variable weakness of active shoulder and elbow flexion movements [7]. Elbow extension, wrist and hand function are additionally impaired when the C7, C8, T1, medial trunk and inferior trunk are involved. An isolated injury of the lower brachial plexus (C8-T1) is rare.

The first focus for therapy is on the type of injury of the peripheral nerve, for that matter, to the severity of the traction injury which may extend from minimal (axonotmesis) to severe (neurotmesis and avulsion) traction, as addressed in previous theses [8, 9]. However, little attention has been paid to the effects on the end organ of the nerve: the muscle and its sequelae on functionality for the patient. A large variety of outcome measures have been used to evaluate the natural history and the effect of treatment, however there is no consensus on which outcome measures are the most appropriate [10, 11]. The International Classification of Functioning, Disability and Health (ICF) is a worldwide accepted model providing a universal language for the description of functioning and includes the domains Body Structures, Body Functions, Activities and Participation as well as Personal and Environmental Factors [12]. ICF Core Sets are generally used to describe the typical spectrum of problems of functioning and health patients with a specific condition (e.g. brachial plexus injury). An important base for the optimal management of OBPI is an in-depth understanding, systematic consideration and sound measurement of the impact of OBPI on health and health-related domains of these patients. To date however, no universally accepted overall framework is available to assess the outcome of patients with (obstetric) BPI.

Muscle degeneration

Long-term denervation results in muscle degeneration including muscle atrophy, fatty degeneration and interstitial fibrosis in the muscle. Current literature on muscle degeneration in the upper extremity of BPI only focuses on total muscle cross sectional area (CSA) and on a qualitative assessment of muscle fatty degeneration including the Goutallier score as a surrogate for contractile CSA, which is the true functional part of the muscle [13-20]. The inter-observer

reliability of the Goutallier score is moderate even in experienced hands [21, 22]. Furthermore, qualitative assessment of fatty degeneration is less sensitive in detecting small differences [23 24, 25]. Quantitative measurements which assess the decrease in contractile CSA compared to the sound extremity could improve insight in the extent of muscle degeneration and would thus facilitate a better treatment strategy.

Muscle denervation and subsequent muscle degeneration results in functional limitations, contractures and osseous deformities. With respect to the elbow, the main complication occurring during follow-up is a flexion contracture, with a prevalence of 50 to 90% [26, 27]. Flexion contractures limited to 10° to 30° can be treated by range of motion exercises and nighttime splinting. In a minority of cases, however, if the contracture exceeds 30°, additional treatment is needed such as serial casting [26, 27]. Although serial casting is frequently applied and globally considered to be the preferred therapy, literature is limited on the effect of stretching by serial casting for elbow contractures. Other clinical consequences of muscle degeneration around the elbow include supination contractures and limited active elbow flexion for which surgical procedures are being performed including forearm osteotomies, biceps rerouting and Steindler elbow flexorplasty [28-33].

Around the shoulder, muscle degeneration often results in internal rotation contractures, with a subsequent posterior humeral subluxation in the growing child, glenoid retroversion and glenoid and humeral head malformation [16, 17, 34]. The prevalence of internal rotation contractures can be as high as 39% depending on the extent and severity of the BPI [35, 36]. Muscle degeneration is most prominent in the subscapular muscle [16, 17]. Treatments of internal rotation contractures include surgical subscapular release combined with transfer of the latissimus dorsi and/or teres major tendon to the rotator cuff to create active external rotation [37-39]. Disadvantages of subscapular release and/or tendon transfer include weaker adduction and potential partial power loss of internal rotation with a subsequent risk for an external rotation contracture of the shoulder. Therefore, coracohumeral ligament releases have been advocated by our group. An even less invasive method to address this internal rotation contracture of the shoulder is the injection of botulinum toxin A (BTX-A) [40, 41]. There have been some reports on BTX-A injections but no clear conclusions could be drawn from these studies since heterogeneity of included patients as well as technique were large (i.e. number and units of BTX-A injections, variety of muscles, combination with tendon transfer surgery etc.) [42-47]. Other surgical procedures to improve shoulder functionality of BPI patients include arthodesis of the shoulder, glenoid anteversion osteotomies and humeral rotation osteotomies [48-51].

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Muscle regeneration

The regenerative potential of skeletal muscle is determined by muscle stem cells, which are called satellite cells. These are quiescent mononucleated cells that are sequestered between the basal lamina and the plasma membrane of the myofibers and can be identified by the expression of the paired box transcription factor Pax7 [52, 53]. The number of satellite cells in adult human has been shown to range from 7% in young age (20 years old) to 1% of all skeletal muscle nuclei in old age (73 years old) [54]. In response to injury, they become activated, proliferate, differentiate and fuse to existing muscle fibers or fuse together to form new myofibers during regeneration of damaged skeletal muscle [55]. This regenerative potential is influenced by replicative and stress-induced premature senescence and apoptosis of these cells.

Replicative senescence is indicated by exhaustion of the pool of available satellite cells and their proliferative capacity which is limited by the mitotic clock [56-58]. Progressive erosion of telomeres after each cell division leads to critically short telomere length and the activation of replicative senescence through a p53 and p21 dependent pathway [59]. The erosion of telomeres can be prevented by the catalytic subunit of the human telomerase reversed transcriptase (hTERT) leading to extension of replicative life [60]. Furthermore, lack of differentiation may contribute to poor functional recovery of long-term denervated muscle [61, 62]. Up-regulation of the p16 dependent pathway results in proliferative arrest before telomeres reach their critical length known as stress induced premature senescence [63, 64]. The regenerative potential of satellite cells is also limited by the extent of apoptosis. Upon denervation, the susceptibility of satellite cells to apoptosis has been shown to increase [65, 66]. Satellite cell activity can be modulated by a microenvironment inducing inflammatory cytokines, however underlying factors influencing the regenerative potential of satellite cells have not yet been identified [58, 67].

Cell therapy has the goal to repair damaged cells and to replenish the exhausted satellite cell pool by (systemic or local) injection of cells with myoregenerative properties. Transplantation of primairy satellite cells has been shown to improve the properties of reinnervated skeletal muscles [68]. However, poor cellular survival and limited cell dissemination hampers successful satellite cell transplantation. Furthermore, only few transplanted cells fuse with host muscle cell fibers. This suggests that a subpopulation of myogenic cells (i.e. stem cells) may be optimally suited for transplantation [69, 70]. Bone marrow (BM)-derived cells migrate to the site of muscle injury and contribute to the satellite cell pool [71-73]. The injection of autologous BM-derived mononuclear cells (MNCs) has been applied in clinical studies focusing on the muscles of the heart and leg [74-77]. Cell therapy could potentially regenerate partially denervated muscle in BPI by replenishment of the satellite cell pool and re-establishment of vascular and neural connections which are essential for muscle growth and function [78, 79].

AIM OF THIS THESIS

The aim of this thesis is to evaluate determinants of outcome, which will have an effect on overall functionality of the patient with a BPI. Deterioration of functionality will either occur immediate after the injury, but will also occur years after the initial peripheral nerve injury. They are generally, but not exclusively, related to the primary target organ of the nerve: the muscle. Secondary to these impaired muscles with subsequent impaired movement of the extremities, joint development in the growing child will be affected. This will have effect at the functionality level of the patient. To this end, outcome measures of functionality using the ICF model are developed (chapter 2). At the clinical level, muscle and joint deformities and their treatment options are addressed (chapter 3, 4, 5). At the cellular level, a deteriorated muscle is characterized in both inflammatory as well as nerve injury patients (chapter 6, 7). Finally, a possible treatment option with cell therapy for this muscle in nerve injury patients is used (chapter 8). The results of this thesis are summarized and future perspectives of muscle degeneration and regeneration for patients with BPI are considered in the discussion (chapter 9).

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Chapter 2

Developing core sets for patients with obstetric brachial plexus injuries based on the International Classification of Functioning, Disability and Health

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ABSTRACT

Background: Symptoms of obstetric brachial plexus injury (OBPI) vary widely over the course of time and from individual to individual and can include various degrees of denervation, muscle weakness, contractures, bone deformities and functional limitations. To date, no universally accepted overall framework is available to assess the outcome of patients with OBPI. The objective of this paper is to outline the proposed process for the development of International Classification of Functioning, Disability and Health (ICF) Core Sets for patients with an OBPI.

Methods: The first step is to conduct four preparatory studies to identify ICF categories important for OBPI: a) a systematic literature review to identify outcome measures, b) a qualitative study using focus groups, c) an expert survey and d) a cross-sectional, multicentre study. A first version of ICF Core Sets will be defined at a consensus conference, which will integrate the evidence from the preparatory studies. In a second step, field-testing among patients will validate this first version of Core Sets for OBPI.

Discussion: The proposed method to develop ICF Core Sets for OBPI yields a practical tool for multiple purposes: for clinicians to systematically assess and evaluate the individual's functioning, for researchers to design and compare studies, and for patients to get more insight into their health problems and their management.

INTRODUCTION

An obstetric brachial plexus injury (OBPI) is caused by traction on the brachial plexus during delivery and can result in severe disabilities in arm function. The incidence varies between 0.4 and 2.9 per 1000 live births [1-3]. The severity of the injury of the brachial plexus may vary from neuropraxia and axonotmesis to neurotmesis and avulsion of rootlets from the spinal cord. The upper part of the brachial plexus is most often affected, resulting in variable weakness of the shoulder and elbow flexion [4]. Elbow extension, wrist and hand function are additionally impaired when the C7, C8, T1, medial trunk and inferior trunk are involved. An isolated injury of the lower brachial plexus (C8-T1) is rare. Depending on the severity and extent of the OBPI, the overall quality of life of patients is very much affected due to impaired upper-limb function. Symptoms of OBPI can vary widely between individuals and clinical studies have been performed to identify the natural history and to improve treatment [5-13]. A large variety of outcome measures is used to evaluate the natural history and the effect of treatment, however there is no consensus on which outcome measures are the most appropriate [14, 15].

An important base for the optimal management of OBPI is an in-depth understanding, systematic consideration and sound measurement of the impact of OBPI on health and health-related domains. The International Classification of Functioning, Disability and Health (ICF) is a worldwide accepted model providing a universal language for the description of functioning and includes the domains Body Structures, Body Functions, Activities and Participation as well as Personal and Environmental Factors (figure 1) [16]. These ICF domains, which contain together more than 1400 categories, can serve as a reference for purposes in the clinical practice [17, 18]. ICF Core Sets are generally agreed on lists of ICF categories and contain as few as possible but as much as necessary ICF categories to describe the typical spectrum of problems of functioning and health of patients with a specific condition. To date, ICF Core Sets have already been or are being developed for more than 20 health conditions, including neurological, musculoskeletal and cardiovascular diseases [19-25]. However, ICF Core Sets for OBPI are not available so far. The objective of this paper is to outline the proposed developmental process for internationally accepted, evidence-based, reliable and valid ICF Core Sets for OBPI.

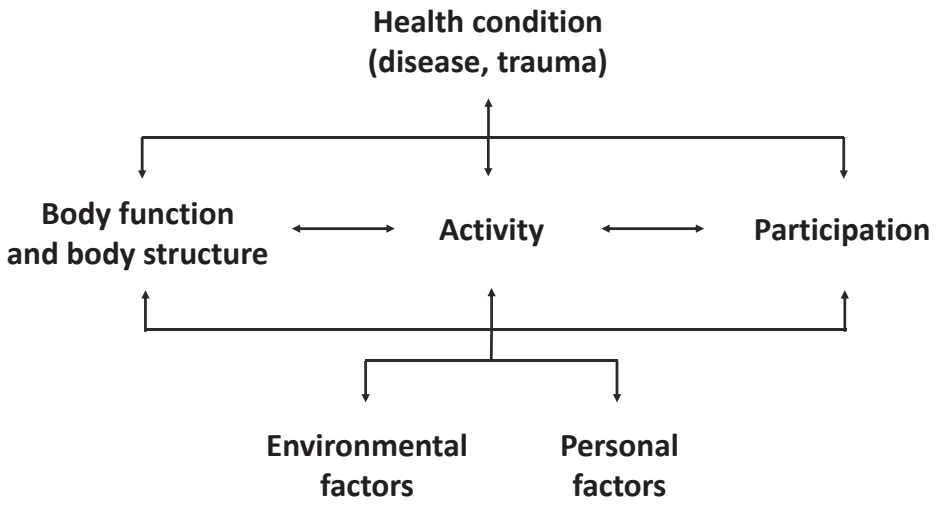

Figure 1: The International Classification of Functioning, Disability and Health of the World Health Organization
Diagram showing the International Classification of Functioning, Disability and Health (ICF) model domains. 'Health condition' is an umbrella term for disease, disorder, injury or trauma and may also include other circumstances, such as aging, stress, congenital anomaly, or genetic predisposition. It may also include information about pathogeneses and/or aetiology. 'Body function' is defined as the physiological functions of body systems, including psychological functions. 'Body structure' refers to the anatomical parts of the body, such as organs, limbs and their components. 'Activity' is the execution of a task or action by an individual and represents the individual perspective of functioning. 'Participation' refers to the involvement of an individual in an everyday situation and represents the societal perspective of functioning. 'Environmental factors' make up the physical, social and attitudinal environment in which people live their lives. 'Personal factors' are the particular background of an individual's life and living situation, and comprise features that are not part of a health condition, such as gender, age, race, fitness, lifestyle, habits, and social background. They can be referred to as those factors that define the person as a unique individual.

METHODS

The development of the ICF Core Sets for OBPI is a collaborative project of the Leiden University Medical Center (LUMC) and the ICF Research Branch of the Collaboration Centre of the Family of International Classifications (DIMDI) at the Ludwig-Maximilian University (Munich, Germany). Figure 2 shows the three phases in the development of the ICF Core Sets for OBPI: the preparatory phase with the international consensus conference (phase I) and the testing and implementation phase (phase II). A summary of the methods is shown in Table I. The project will be conducted in conformity with the ethical principles of Declaration of Helsinki. All appropriate study-related documents will be presented to the Medical Ethics Committees for review and approval.

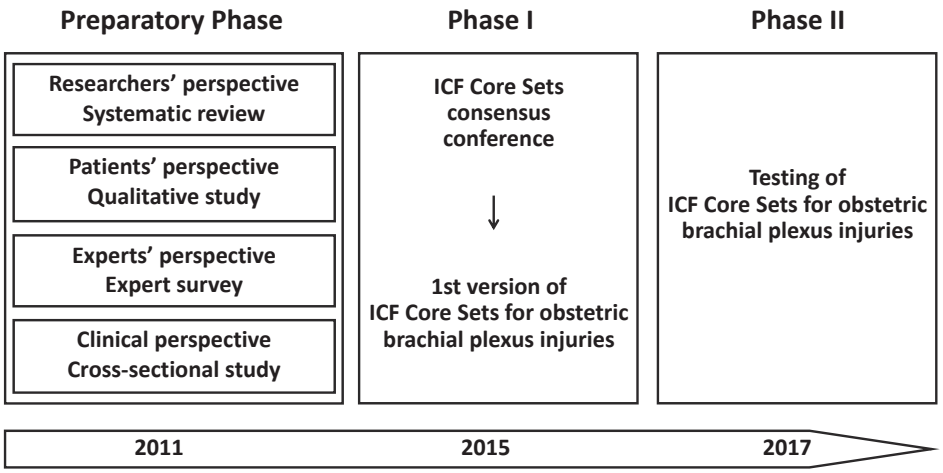

Figure 2: Time schedule and phases of the project

Preparatory phase

The preparatory phase aims at identifying concepts relevant to patients with OBPI from 4 different points of view. Therefore, this phase consists of 4 different preparatory studies: a systematic review, a qualitative study, an expert survey and a cross-sectional study.

Systematic review: The objective of this study is to capture the researchers' perspective (a) by identifying outcome measures cited in published studies with the focus on individuals with OBPI and (b) by identifying and quantifying the concepts contained in these measures using the ICF as a reference. Electronic databases regarding the measures used in clinical trials and in observational studies identifying concepts from the researchers' point of view. The data collection process consists of three subsequently steps: Firstly, the parameters used in published studies are identified; secondly the items of all retrieved parameters and their underlying concepts are specified. Finally, these concepts will be linked to the categories of the ICF using standardized linking rules [26, 27]. Absolute and relative frequencies of the parameters and the linked ICF categories will be reported.

Qualitative study: The objective of this study is (a) to explore and understand the perspective of patients with OBPI on functioning and health and (b) to identify and quantify the concepts of functioning and health important to patients with OBPI using the ICF as a reference. It is important to include the patients' perspective because personal values for outcomes vary between and within patients. To identify

Table I: Summary of the methods for the development of International Classification of Functioning, Disability and Health (ICF) Core Sets for obstetric brachial plexus injury (OBPI) (*continued*)

	Methods	Objectives	Specifics
Systematic review	A systematic review uses explicit methods to systematically search, critically appraise, and synthesize the world literature on OBPI	(a) To identify outcome measures cited in published studies with the focus on patients with OBPI (b) To identify and quantify the concepts contained in these measures using the ICF as a reference	To obtain the researchers perspective, electronic databases will be search using keywords including "Brachial Plexus Neuropathies", "Brachial Plexus injuries", "Erb Paralysis", "Klumpke Paralysis" and "Obstetric Brachial Plexus"
Qualitative study	Focus group technique is a qualitative method valid to explore the perspective of those who experience OBPI, i.e. the so-called patient perspective	(a) To explore and understand the perspective of patients with OBPI on functioning and health (b) To identify and quantify the concepts of functioning and health important to patients with OBPI using the ICF as a reference	To obtain an international perspective, focus groups are conducted with people with OBPI in different countries
Expert survey	A survey involving experts experienced in the treatment of individuals with OBPI	(a) To identify the most relevant problems of patients with OBPI from the perspective of clinical experts (b) To identify and quantify the concepts contained in these problems using the ICF as a reference	To obtain a worldwide perspective, experts from all over the world and from different health professions will be included
Cross-sectional study	A multicenter, cross-sectional study that involves data collection at one time point	(a) To describe the prevalence of problems in functioning of patients with OBPI (b) To identify the categories that explain most of the variance of external standards	To obtain an international perspective, centers in different countries will be involved
Consensus conference	The "Nominal-Group Technique" is a consensus-planning process that helps to prioritize issues during an expert's conference	To develop ICF Core Sets for OBPI in a formal decision making and consensus process, integrating evidence gathered from preliminary studies and expert opinion	The number of groups is set at 4, always including 7 experts to address the main interests and to involve the different professions and WHO regions. Each group will consist of different health professionals who work together in a multi-professional and multi-disciplinary approach

Table I: Summary of the methods for the development of International Classification of Functioning, Disability and Health (ICF) Core Sets for obstetric brachial plexus injury (OBPI) (*continued*)

	Methods	Objectives	Specifics
Implementation and testing	Worldwide Empirical studies, including external standards, like the Short Form-36	To study the content validity and feasibility of the Comprehensive and the Brief ICF Core Sets for OBPI	A network of organizations and institutions from all over the world which are interested in the implementation and testing of the pilot ICF Core Sets will be established

the prototypical spectrum of problems of patients with OBPI small groups with up to eight individuals, between the age of eight and eighteen years, are interviewed. These focus groups are likely to generate more concepts then individual interviews because the interaction and group process can enrich the information generated within a group of patients [28, 29]. A topic guide based on the components Body Structures, Body Functions, Activities and Participation, Environmental Factors and Personal Factors of the bio-psycho-social model will be applied. All interviews will be digitally recorded, transcribed verbatim and evaluated again by two independent persons. Participants will be included in the study until saturation is reached which is defined as the point during data collection when the linking of the retrieved concepts to the 2nd level of the ICF classification is less than 5% of the number of 2nd level ICF category nominated so far. The frequencies of the parameters and the linked ICF categories from the focus groups will be reported.

Expert survey: An Internet based expert survey will be conducted (a) to collect the health professionals' perspective on relevant problems of patients with OBPI and (b) to identify and quantify the concepts contained in these problems using the ICF as a reference. Since the ICF was developed to facilitate communication between different groups of people and to be used globally, the aim is to include experts from all six WHO regions: Eastern Mediterranean, South-East Asia, Western Pacific, The Americas, Africa and Europe. To maximize the number and range of ideas and opinions, the aim is to include experts from different health professions including neurosurgeons, orthopedic surgeons, plastic surgeons, rehabilitation physicians, neonatologists, neurologists, pediatricians, physical therapists, occupational therapist, nurses and social workers. The health professionals have to be experienced in the treatment of OBPI for at least two years and have to be fluent in English to contribute to the survey. In the expert survey a 5 item open-ended questionnaire is used which requests that experts list all body structures, functions,

activities and participation aspects in which patients with OBPI encounter problems as well as all contextual factors which have a facilitation and/or hindering influence on the problems of patients with OBPI. The answers of the health professionals are linked to the ICF categories and the frequency of ICF categories are reported.

Cross sectional study: To obtain the clinical perspective, a multi-center cross-sectional study will be conducted. The aims of this study are (a) to describe functioning and health of patients with OBPI, (b) to identify the most common problems using the ICF and (c) to identify the categories that explain most of the variance outcome measures currently used. This multi-center study will be conducted in the different WHO regions and patients with OBPI who are literate in the primary language of the study site will be consecutively included in the study. Data will be collected with a case record form (CRF), which is made up of two parts. The first part is an ICF checklist, which consists of the 31 1st level categories and a selection of 123 from the in total 362 2nd level categories of the whole ICF classification system. It provides a relatively simple questionnaire, which can be filled out by the physician or other health professional. The ICF checklist makes it possible to classify the most important ICF categories in clinical practice. The second part includes the EuroQol (EQ-5D) [30], five questions of the WHO Quality of Life Questionnaires (WHOQoL) to assess the subjective appraisal of health and well-being [31], the SF-36 Health Survey [32], the Disability of Arm, Shoulder and Hand Questionnaire (DASH) [33], and the self-administered Co-morbidity Questionnaire (SCQ) [34]. Furthermore, sociodemographic variables will be collected. For the ICF components body functions, body structures, activities and participation, absolute frequencies and relative frequencies of impairment will be reported along with their 95% confidence intervals. For environmental factors, absolute frequencies and relative frequencies of persons who regarded a specific category as either barrier or facilitator are reported.

Consensus conference

The results of the preparatory phase will be presented at an international consensus conference. At this conference, experts in the field of OBPI will work actively together to deal with the complex problem of arriving at an international consensus on the most adequate ICF categories to be included on the ICF Core Sets for OBPI. The "Nominal-Group Technique" will be applied to regulate the group dynamic and the teamwork during the conference [35]. Since the results of the preliminary studies are all expressed in ICF language, they can be compiled in tables that contain for each category addressed the percentage with which the categories were named in the preparatory studies. First, the experts will be asked to select ICF categories for the comprehensive ICF Core Sets, which is a list of

ICF categories long enough to describe the prototypical spectrum of functioning and health of patients with OBPI. Second, the experts will be asked to select from the comprehensive ICF Core Sets lists the ICF categories, which are enough to describe the prototypical spectrum of functioning of patients with OBPI, but this list needs to be short enough to be feasible to use in clinical practice. This process will lead to a brief list of ICF Core Sets.

Validation phase

The next step is to test the validity and feasibility of the proposed brief and comprehensive ICF Core Sets for patients with OBPI. This will be conducted in a multicenter cohort study at a network of cooperating organizations. The objectives are to analyze the frequency of patients' problems in different subsets, including nationality, socioeconomic factors, age, gender, the severity of brachial plexus injury and co-morbidities. The results will be used to identify the categories that explain most of the variance of external standards, as well as to analyze to what extent the entire severity spectrum of problems in functioning reported by patients with OBPI is represented in the comprehensive ICF Core Sets.

DISCUSSION

In this paper we have described the proposed process to develop ICF Core Sets for OBPI. The ICF has been proposed by the World Health Organization to describe comprehensively limitations in functioning and relevant environmental factors [16]. The ICF offers a framework and classification for the patient and clinical perspective both at the individual and the population level. It integrates long known concepts and provides a universal and standardized language. ICF Core Sets are necessary to make the ICF useful to describe functional limitations and disabilities of patients with OBPI.

Currently, ICF Core Sets have been, or are being developed for more than 20 health conditions, including neurological traumas and musculoskeletal diseases like traumatic brain injuries, spinal cord injuries, osteoarthritis and low back pain [19-25]. ICF Core Sets may well serve as the basis for multi-professional patient assessment, goal setting, intervention management and evaluation [36]. For a correct interpretation of the patient's problems, it is important to first describe the concerns of the patient and/or parents in their own words. Physicians can use the ICF Core Sets to describe and classify patients' symptoms and findings of clinical examinations. Professionals could describe at least all categories from a brief Core Set and use the comprehensive Core Set as a source of additional relevant items. Using the ICF Core Sets supports multidisciplinary and comprehensive assessment of functioning. It helps all team members to consider every potentially relevant

aspect of functioning of a patient. The use of ICF Core Sets can assist health care professionals in explaining the necessity of a certain procedure for a patient and for their parents thereby creating more insight into the health problems and treatment. It is by no means the aim of the ICF to replace validated existing instruments. In contrast, it is likely that they will have an essential role to measure particular parts of the ICF. Most measurement instruments do however cover only a limited number of ICF categories.

Next to the clinical application, ICF Core Sets will be used by researchers and healthcare providers in many ways. ICF Core Sets make it possible to classify and describe an individual's functioning using widely accepted terminology, which facilitates comparability of international studies. It can also be used to rate the content validity of health-status measures and to select appropriate instruments for the specific needs for patients with OBPI. In the future, ICF and the ICF Core Sets may become the new basis not only for the further development of such measures, but also for the creation of an item list with parameters relevant to patients with OBPI. Furthermore, ICF facilitates linking medical data across different conditions or interventions for efficient, transparent and cost-effective health care. Other benefits could also rise from compiling a functionality assessment for OBPI from a common set of indicators (a common bio-psycho-social framework) used for all health conditions. For example, a common language would facilitate communication among health professionals and also between health professionals and patients with OBPI, as well as their family caregivers. In addition, because the ICF is etiologically neutral, it is easily possible to combine the ICF Core Sets for OBPI with other ICF Core Sets in a non-redundant and useful way when describing and classifying functioning and disability of persons with more than one condition. Worldwide comparisons will also be possible for any health condition, not only for OBPI. This will enable policy makers to make informed decisions with regard to such issues as resource allocation, new legislation and modification of care provision policies.

A few limitations have to be resolved during the developmental process of ICF Core Sets. The assessment of functioning and quality of life data is highly sensitive to ethno-cultural differences [37, 38]. In order to come up with a first version of ICF Core Sets for OBPI that integrates information from different cultural backgrounds, the preparatory studies will gather data on patients being treated at centers from different countries and from professionals in an international expert survey. Another limitation could be that the preparatory studies collect information only from published papers in English and English speaking participants. This is done to ease comparability of results from different countries, but on the other hand will contain a structural bias for countries where English is not the native language.

The extent and relevance of this issue will be addressed in the validation phase following the consensus conference. An issue to be addressed is the possible need for stratification by a number of variables including the extent of brachial plexus injury and the age of the growing children. The preliminary studies will provide the necessary information to guide these decisions. It is important to realize that the ICF Core Sets Consensus Conference will provide only a first, best possible version of ICF Core Sets for OBPI, which will then need to be tested worldwide.

In conclusion, this is a big step forward in the development of an internationally acceptable and standardized tool for assessing disability in OBPI. Establishing standardized ICF Core Sets will be useful for research, clinical practice and teaching. The ICF Core Sets will then also form the basis for the development of assessment instruments to quantify the severity of OBPI to measure change over time and the effectiveness of interventions. They could also serve as the basis for setting clinical significance thresholds in diagnostic assessment systems. Finally, it is our hope that such research will lead to interventions and accommodations that improve the restoration and maintenance of functioning and minimize disability among patients with OBPI throughout the world.

INVITATION FOR PARTICIPATION

The development of the ICF core sets is an inclusive and open process. Therefore, the authors of this paper encourage clinical experts and patients to actively participate in the process. Anyone who wishes to actively participate is invited to contact the Leiden ICF brachial plexus team (brachialplexus@lumc.nl). Individuals, institutions and associations can be formally associated as partners of the project.

AUTHOR'S CONTRIBUTIONS

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Formation of ICF Brachial Plexus Advisory Board: *Saraç, Malessy, Vliet Vlieland, Nelissen*
First draft of the manuscript: *Duijnisveld, Vliet Vlieland*
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Chapter 3

Quantitative Dixon and qualitative T1 MRI sequences to relate muscle atrophy and fatty degeneration with range of motion and muscle force in brachial plexus injury

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ABSTRACT

Background: Assessment of muscle atrophy and fatty degeneration in brachial plexus injury (BPI) could yield valuable insight into pathophysiology and could be used to predict clinical outcome. The objective of this study was to quantify and relate fat percentage and cross-sectional area (CSA) of the biceps to range of motion and muscle force of traumatic brachial plexus injury (BPI) patients.

Methods: T1- weighted TSE sequence and three-point Dixon images of the affected and non-affected biceps brachii were acquired on a 3 Tesla magnetic resonance scanner to determine the Goutallier score, fat percentage, total and contractile CSA of 20 adult BPI patients. Regions of interest were drawn by two independent investigators to determine the inter-observer reliability. Paired Students' t-test and multivariate analysis were used to relate fat percentage, total and contractile CSA to active flexion and biceps muscle force.

Results: The mean fat percentage 12 ± 5.1% of affected biceps was higher than 6 ± 1.0% of the non-affected biceps ($p < 0.001$). The mean contractile CSA 8.1 ± 5.1 cm^2 of the affected biceps was lower than 19.4 ± 4.9 cm^2 of the non-affected biceps ($p < 0.001$). The inter-observer reliability was excellent (ICC 0.82 to 0.96). The Goutallier score was strongly associated with fat percentage (Spearman's rho 0.87, $p < 0.001$), however it gave an overestimation in those classified with a high grade Goutallier. The contractile CSA contributed most to the reduction in active flexion and muscle force.

Conclusion: Quantitative measurement of fat percentage, total and contractile CSA using three-point Dixon sequences provides an excellent reliability and relates with active flexion and muscle force in BPI.

INTRODUCTION

Brachial plexus injury (BPI) results in severe nerve damage affecting the upper extremity. Despite partial natural recovery, nerve and/or secondary surgery, both traumatic BPI patients and neonatal brachial plexus palsy patients do not regain normal upper extremity function and are impaired in muscle force and range of motion of the shoulder, elbow, wrist and/or hand [1-7]. Long-term denervation results in muscle degeneration including muscle atrophy, fatty degeneration and interstitial fibrosis in the muscle. Quantitative tools which assess the decrease in the amount of muscle tissue could improve insight in the extent of muscle degeneration and could facilitate a better treatment strategy. The current literature on muscle degeneration in the upper extremity of BPI focuses on total muscle cross sectional area (CSA) and on a qualitative assessment of muscle fatty degeneration using the Goutallier score on T1 weighted Turbo Spin Echo (TSE) magnetic resonance (MR) images as well as at computed tomography (CT) scans [4, 7-13]. The current literature has limitations as the inter-observer reliability of the Goutallier score is moderate even in experienced hands [14, 15]. Furthermore, a qualitative assessment of fatty degeneration is less sensitive in detecting small differences compared to a quantitative assessment [16-18]. Finally, an overall qualitative muscle score (i.e. Goutallier score) and assessments of total muscle CSA measure both fatty degeneration as well as contractile muscle tissue, whereas only the latter is the true functional part of the muscle [4, 7-13].

The three-point Dixon sequence can be applied to quantify intramuscular fat (i.e. indirect contractile muscle tissue). This sequence uses a chemical shift based approach which relies on the difference in resonance frequency between water and fat. Previously, the Dixon sequence has been used extensively to measure intramuscular fat in different conditions, including rotator cuff tears [19, 20], lean and obese children [21], diabetes [22, 23] and the muscular dystrophies [17, 18, 24-30]. However, the extent of muscle fatty degeneration as an indirect measure and the amount of contractile tissue in BPI is currently unknown.

The first objective of this study was to quantify intramuscular fat, the total and contractile CSA in BPI patients and to assess the inter-observer reliability and the variability using three-point Dixon MRI. The second objective was to correlate the qualitatively assessed intramuscular fat with the Goutallier score on a T1 weighted TSE sequence with the quantitatively obtained value. The final objective was to assess whether intramuscular fat, the total and contractile CSA were associated with elbow range of motion and muscle force in severely affected BPI patients.

METHODS

Patients

An observational study was performed including 20 adult BPI patients recruited from the peripheral nerve injury unit of the Leiden University Medical Center. Inclusion criteria were an age > 18 years old and a traumatic BPI. To create a uniform group of patients who are at the end stage of neural regeneration, all patients had to be minimum two years after trauma and/or nerve surgery for the biceps muscle. Patients were excluded if they had a fracture of the humeral bone, bilateral brachial plexus lesion, secondary surgery around the shoulder or elbow, or contra-indications for MRI. The medical ethical review board of the Leiden University Medical Center approved the protocol of this study and all patients signed informed consent. This study was registered in the Netherlands Trial Register, number NTR2524.

MRI

MR imaging was performed on a 3 Tesla MR machine (Philips Achieva, Philips Medical Systems, Best, the Netherlands) in supine position, with the arm as much in the center of the magnet bore as possible and the patient's arms placed alongside of the body with the thumbs directed upwards. Both arms were imaged separately. A 14-cm two-element receive coil was used for signal reception. The receive coil elements were positioned on the anterior and posterior side in the middle of the upper arm using the humeral head and olecranon as palpable bony reference. The scan protocol consisted of axial T1-weighted TSE sequence (16 slices of 7.5 mm thickness, 0.75 mm gap, repetition time (TR) 600 ms, echo time (TE) 16 ms, field of view (FOV) 180 x 180 mm, voxel size 0.6x0.6 mm^2, TSE factor 5, acquisition time 5:20 minutes) and a 3-point gradient echo Dixon sequence (16 slices of 7.5 mm thickness, 0.75 mm gap, TR 400 ms, first TE 4.41 ms, echo spacing 0.76 ms, flip angle 8°, FOV 180x180 mm, (voxel size 0.9x0.9 mm^2), acquisition time 6:30 minutes) of the affected and non-affected upper arm. The T1 and Dixon sequences were planned using a survey scan, around the distal 2/3 of the humeral bone using the humeral head and epicondyles of the distal humeral bone as bony landmark. Representative examples of T1, Dixon fat and Dixon water images of the non-affected and affected arm are shown in figure 1.

Data analysis

The three-point Dixon images were reconstructed with multipeak correction as described before, with frequencies f_p = [94, -318, -420] Hz and amplitudes A_p = [0.08, 0.15, 0.78], to account for the multiple peaks of the fat spectrum [28]. No corrections were made for partial saturation due to T1, T2 or T2* relaxation. Regions of interest

(ROIs) were drawn manually on every slice by two independent investigators (B.J.D. and J.F.H.), blinded for patient details using the Medical Image Processing, Analysis and Visualization software package (http://mipav.cit.nih.gov) on T1-weighted images in the biceps and triceps brachii of the affected and non-affected arm. Five consecutive slices with the largest cross sectional area of the biceps and triceps brachii were used to calculate the mean fat percentage, the total CSA (i.e. the CSA including muscle tissue and intramuscular fat tissue), and the contractile CSA which was calculated from the mean fat percentage and the total CSA (contractile CSA = total CSA (100 - mean fat percentage) / 100). The mean fat percentage per muscle was computed using the co-registered contours from the T1 weighted images on the Dixon images and calculated by averaging all pixels assigned to that muscle. Next, the mean fat percentage was calculated by: signal intensity on fat image / (signal intensity on fat image + signal intensity at water image). The Goutallier grading was scored by two experienced musculoskeletal radiologists (CSPR and MR) in consensus: 0 'no fat', 1 'some fatty streaks', 2 'less fat than muscle', 3 'as much fat as muscle' and 4 'more fat than muscle'. Furthermore, the affected biceps and triceps brachii were scored for the presence of atrophy compared to the non-affected biceps and triceps brachii of the contra lateral arm.

Clinical parameters

The age, gender, affected side, severity of the lesion according to Narakas and type of nerve and/or secondary surgery was recorded [31]. The passive and active elbow range of motion (flexion, extension, supination and pronation) was measured using a hand held goniometer. Muscle force was measured (semi) quantitatively of both elbow flexion and elbow extension using the medical research council (MRC) scale and a hand-held dynamometer (MicroFET2, Biometrics, Almere, the Netherlands) in a standardized arm positions (90° flexion and 90° supination). Muscle quality was determined by calculating the specific muscle force i.e. muscle force in Newton per cm^2 of contractile CSA. The self assessment questionnaires disability of the arm, shoulder and hand (DASH), the short form-36 (SF-36) and visual analogue score (VAS) for pain (0-10) were used to assess the quality of life of the BPI patients.

Statistical analysis

The paired Student's t-test was used to study differences in fat percentage, total CSA, contractile CSA and specific muscle force between the affected and the non-affected biceps and triceps brachii. The intraclass correlation coefficient (ICC) of fat percentage, total and contractile CSA was calculated to determine the reliability between two independent observers and potential variability between 5 consecutive MRI slices using the 2-way random model with absolute agreement [32].

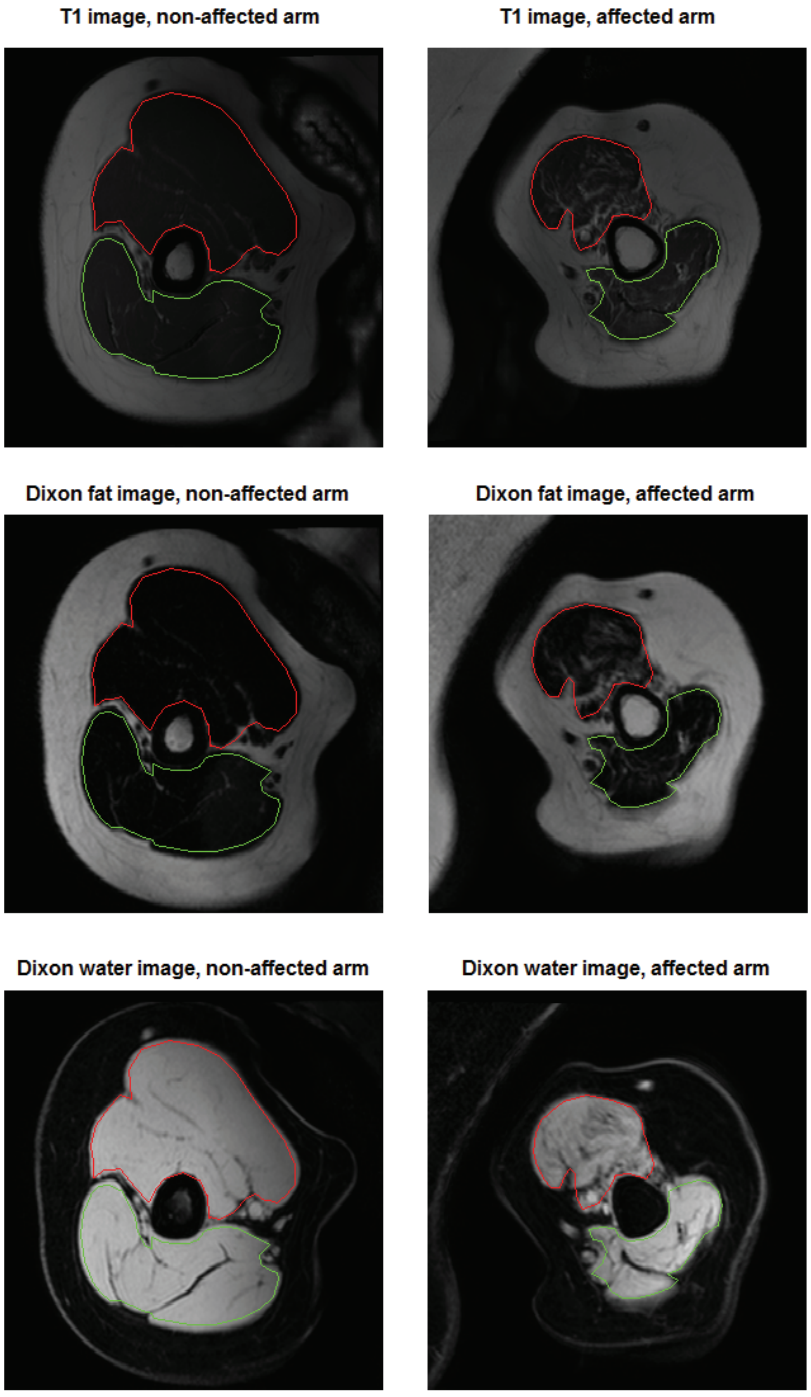

Figure 1: Example of T1, Dixon fat and Dixon water images of the non-affected and affected arm. Regions of interest are drawn in red for the biceps and green for the triceps brachii.

For interpretation, the criteria formulated by Cicchetti and Sparrow were used: 0.00 to 0.39, poor; 0.40 to 0.59, fair; 0.60 to 0.74, good; or 0.75 to 1.00, excellent [33]. The Spearman's rho correlation coefficient was used to investigate the presence of correlation between the percentages of fat using Dixon with the Goutallier score of the T1w images. Univariate linear regression analysis was employed to study the association between fat percentage, total and contractile CSA with range of motion and muscle force in Newton. Logistic regression analysis was used to associate fat percentage, total and contractile CSA to muscle force in MRC 0 to 3 versus MRC 4 and 5. In the multivariate analysis, age and elapsed time since the trauma were used. For statistical analysis a SPSS software package was used (version 23.0, IBM Inc., Armonk, New York, USA). All analyses were two tailed and *p*-values < 0.05 were considered significant.

RESULTS

Patients

The patient characteristics are summarized in table I. In one patient, the non-affected arm could not be scanned due to claustrophobia of the patient after scanning the affected arm. The mean fat percentage was 12 ± 5.1 % in the affected biceps brachii which was significantly higher than 6 ± 1.0 % in the non-affected biceps brachii ($p < 0.001$) as shown in figure 2. The mean fat percentage was 10 ± 4.3 % in the affected triceps brachii, compared to 6 ± 1.6 % of the non-affected triceps brachii ($p = 0.001$). The mean total CSA of the affected biceps brachii was 9.0 ± 5.3 cm^2 which was lower than a mean of 20.7 ± 5.2 cm^2 of the non-affected biceps brachii ($p = 0.001$). The mean contractile CSA was lower in the affected biceps brachii 8.1 ± 5.1 cm^2, compared to a mean of 19.4 ± 4.9 cm^2 in the non-affected biceps brachii ($p < 0.001$). The total and contractile CSA were also lower in the affected triceps brachii compared to the non-affected triceps brachii as shown in table II.

Reliability

The interobserver reliability was excellent for fat percentage, total CSA and contractile CSA in both the biceps and the triceps brachii (table III). To measure the homogeneity between the MRI slices, the ICC of 5 consecutive MRI slices was calculated. The ICC was excellent for fat percentage, total and contractile CSA of the biceps brachii and the triceps brachii (table III).

Table I: Patient characteristics

	N = 20
Sex * (male)	17
Age † (years)	37 ± 11.1
Body mass index† (kg/m^2)	25 ± 3.8
Dominancy before trauma * (right / left / both)	16 / 3 / 1
Brachial plexus injury	
Age at trauma † (years)	31 ± 10.8
Side * (right / left)	10 / 10
Narakas type* (C5-C6 / C5-C7 / C5-C8 / C5-T1)	5 / 1 / 3 / 11
Primary treatment	
Conservative / neurolysis / nerve transplantation *	4 / 1 / 15
Age at neurosurgery † (years)	31 ± 11.3
Type of nerve transplantation *	
Anterior division superior trunk	6
Posterior division superior trunk	3
Medial trunk	1
Suprascapular nerve	2
Musculus cuteneus nerve	8
SF36 questionnaire †#	72 ± 18.1
DASH questionnaire †‡	23 ± 17.6
Employed *#	16
Playing sport / instrument*#	9
VAS for pain †#	3.5 ± 3.06

*The values are given as the number of patients. † Values are given as mean with standard deviation #data was obtained from 19 patients, ‡data was obtained from 18 patients. SF36: Short-Form 36. DASH: Disability of the arm, shoulder and hand. VAS: Visual analogue scale with range from 0 ´no pain´ to 10 ´maximum pain´.

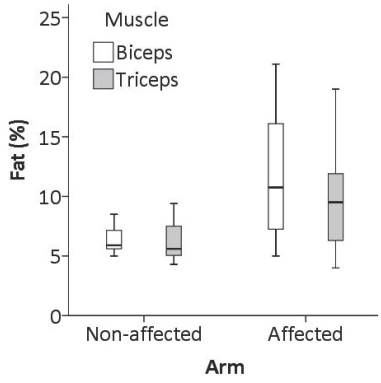

Figure 2: Fat percentage of the affected en non-affected biceps and triceps

Table II: Results of fat percentage, total and contractile CSA in the affected and non-affected biceps and triceps brachii

	Affected arm	Non-affected arm	Mean difference (95 % CI)	*p* - value
Fat (%)				
Biceps brachii	12 ± 5.1	6 ± 1.0	-5 (-3 to -7)	< 0.001
Triceps brachii	10 ± 4.3	6 ± 1.6	-3 (-2 to -5)	0.001
Total CSA * (cm^2)				
Biceps brachii	9.0 ± 5.3	20.7 ± 5.2	-11.5 (-15.0 to -7.9)	< 0.001
Triceps brachii	10.3 ± 6.6)	20.2 ± 4.8	-9.5 (-12.7 to -6.4)	< 0.001
Contractile CSA # (cm^2)				
Biceps brachii	8.1 ±5.1	19.4 ± 4.9	-11.1 (-14.5 to -7.7)	< 0.001
Triceps brachii	9.4 ±6.3	18.9 ± 4.5	-9.1 (-12.1 to -6.2)	< 0.001

*The CSA including muscle tissue and intramuscular fat tissue, #contractile CSA = total CSA (100 - mean fat percentage) / 100, CI: confidence interval, CSA: cross sectional area.

Table III: Intraclass correlation coefficients of 2 independent observers and 5 consecutive MRI slices

	2 independent observers			5 consecutive MRI slices		
	ICC	95 % CI	p-value	ICC	95 % CI	p-value
Muscle fat (%)						
Biceps brachii	0.88	0.73 – 0.94	< 0.001	0.94	0.91 – 0.96	< 0.001
Triceps brachii	0.82	0.65 – 0.89	< 0.001	0.92	0.87 – 0.95	< 0.001
Total CSA * (cm^2)						
Biceps brachii	0.95	0.43 – 0.99	< 0.001	0.99	0.98 – 0.99	< 0.001
Triceps brachii	0.96	0.56 – 0.99	< 0.001	0.95	0.92 – 0.97	< 0.001
Contractile CSA # (cm^2)						
Biceps brachii	0.88	0.36 – 0.96	< 0.001	0.99	0.99 – 1.00	< 0.001
Triceps brachii	0.89	0.45 – 0.96	< 0.001	0.95	0.92 – 0.97	< 0.001

*The CSA including muscle tissue and intramuscular fat tissue, #contractile CSA = total CSA (100 - mean fat percentage) / 100. MRI: magnetic resonance imaging, ICC: interclass correlation coefficient, CI: confidence interval, CSA: cross sectional area.

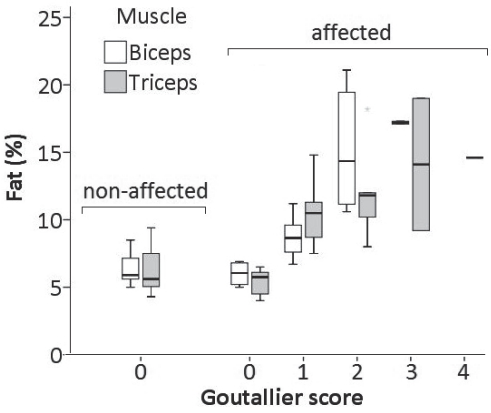

Figure 3: Association of quantitative fat percentage on Dixon with qualitative Goutallier score on T1 of the affected and non-affected biceps and triceps

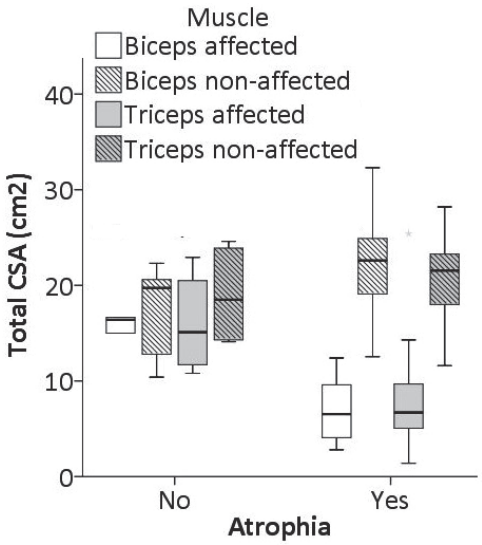

Figure 4: Association of total cross sectional area with atrophy of the affected and non-affected biceps and triceps

Qualitative versus quantitative fat scores

The quantitative fat percentage was strongly associated with the qualitative Goutallier score with a Spearman's rho correlation coefficient of 0.87 for the biceps ($p < 0.001$) and 0.78 for the triceps ($p < 0.001$), as depicted in figure 3. However, the Goutallier score overestimates the fat percentage as patients graded with score 3 or 4 (i.e. fat percentage of 50% or higher) had a three-point Dixon fat percentage range from 9 to 19%. The qualitative score of atrophy was compared to the quantitative score of total CSA, as shown in figure 4. In the patients where the mean total CSA of the biceps brachii was visually scored as 'not atrophic', there was indeed no significant difference between the affected and the non-affected arm (mean difference -1.6 cm^2, 95% CI 4,8 to 1.6, $p = 0.23$). Also the mean total CSA of the triceps brachii which were scored as 'not atrophic', was not significantly different between the affected and the non-affected arm (mean difference -2.9 cm^2, 95% CI -7.7 to 2.0, $p = 0.18$). The mean total CSA of biceps brachii which were scored 'atrophic' was significantly smaller in the affected compared to the non-affected biceps (mean difference -15.0 cm^2, 95% CI -17.7 to -12.3, $p < 0.001$). The same results were observed for the triceps brachii (mean difference -11.9 cm^2, 95% CI -15.1 to -8.7, $p < 0.001$).

Relation to clinical outcome

The association of fat percentage, total and contractile CSA of the affected and non-affected biceps and triceps brachii with muscle force in MRC is shown in figure 5. Clinical results of passive and active range of motion and muscle force are shown in table IV. The specific muscle force was lower in the affected biceps brachii (mean 10 ± 5.4 N/cm^2) compared to the non-affected biceps (mean 16 ± 4.8 N/cm^2) ($p = 0.002$), while this failed to reach significance in the triceps brachii (mean 12 ± 4.9 N/cm^2 in the affected versus 14 ± 3.6 N/cm^2 in the unaffected arm ($p = 0.078$). Univariate and multivariate regression analyses are described in tables V to VII. Multivariate regression analysis showed that contractile CSA of the biceps brachii was most significantly related to several clinical outcome parameters including elbow flexion (7.1°, 95% CI 2.8 to 11.5, $p = 0.003$), supination (5.5°, 95% CI 0.6 to 10.4, $p = 0.030$), muscle force in MRC (odds ratio 2.6, 95% CI 1.1 to 6.1) and muscle force in Newton (13.0N, 95% CI 8.9 to 17.1).

Table IV: Clinical results

	Muscle force of the affected biceps brachii						Non- affected
	MRC 0 N = 3	MRC 1 N = 2	MRC 2 N = 1	MRC 3 N = 2	MRC 4 N = 10	MRC 5 N = 2	Biceps brachii N = 20
Active flexion † (°)	-	-	60	83 ± 10.6	133 ± 15.9	145 ± 7.1	140 (150 – 150)
Active extension † (°)	-	-	-20	-15 ± 7.1	-9 ± 9.7	-5 ± 7.1	0 (0 – 0)
Active pronation † (°)	-	45 ± 64	30	45 ± 63.6	69 ± 33.8	80 ± 14.1	90 (90 – 90)
Active supination † (°)	-	-	-30	-40 ± 56.6	25 ± 54.9	90 ± 0.0	90 (90 – 90)
Passive flexion † (°)	118 ± 18.9	145 ± 7.1	140	133 ± 3.5	143 ± 10.3	150 ± 0.0	143 (150 – 150)
Passive extension † (°)	-11 ± 7.6	-	-20	-15 ± 7.1	-7 ± 8.5	-5 ± 7.1	0 (0 – 0)
Passive pronation † (°)	90 ± 0.0	90 ± 0.0	90	90 ± 0.0	82 ± 16.2	90 ± 0.0	90 (90 – 90)
Passive supination † (°)	50 ± 34.6	15 ± 21	-30	0 ± 0.0	5 ± 37.3	90 ± 0.0	90 (90 – 90)
Force triceps ‡ (MRC)	0 (0 – 0)	2 (0 – 4)	0	0 (0 – 0)	4 (3 – 4)	5 (5 – 5)	5 (5 – 5)
Force biceps † (Newton)	-	-	-	-	90 ± 61.4	193 ± 67.2	306 ± 55.2
Force triceps † (Newton)	-	38 ± 54	-	-	125 ± 93.6	214 ± 48.8	248 ± 63.6
Upper arm circumference † (cm)	23 ± 2.6	21 ± 3.2	26	29 ± 1.4	24 ± 2.5	26 ± 1.4	29 ±2.8

†The values are given as mean with standard deviation. ‡The values are given as median with inter quartile range. MRC: medical research council.

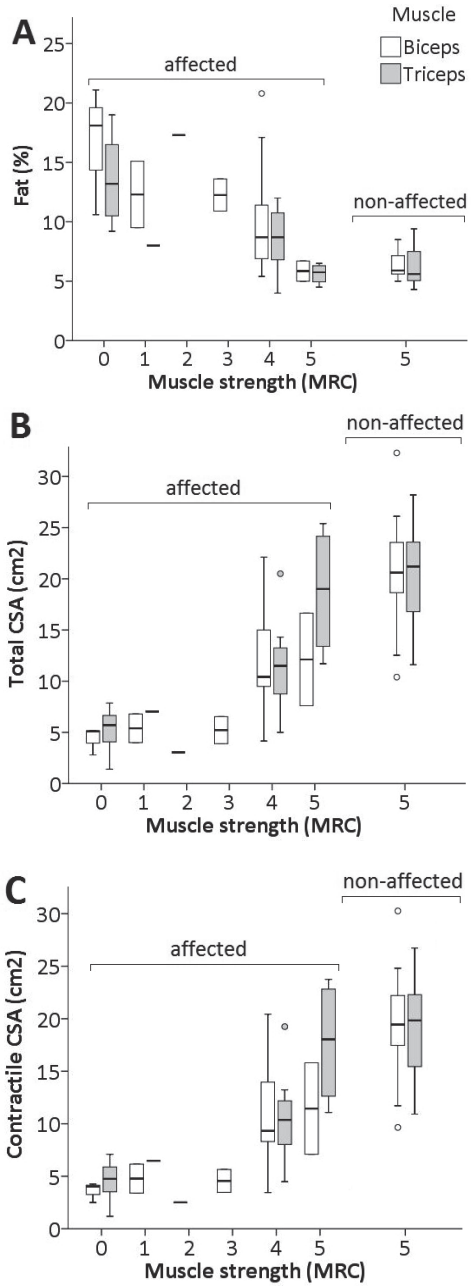

Figure 5: Association of fat percentage (A), total CSA (B) and contractile CSA (C) with muscle force of the affected and the non-affected biceps and triceps

Table V: Linear regression for active range of motion

	Regression coefficient	95% CI	*p* - value
Active flexion univariate models for the affected biceps brachii			
Fat percentage	-6.0	-10.8 to -1.2	0.017
Total CSA *	7.0	2.8 to 11.2	0.003
Contractile CSA #	7.3	2.9 to 11.7	0.003
Active flexion multivariate models for the affected biceps brachii			
Fat percentage	-5.9	-10.9 to -0.8	0.025
Total CSA *	6.8	2.6 to 11.0	0.004
Contractile CSA #	7.1	2.8 to 11.5	0.003
Active extension univariate models for the affected triceps brachii			
Fat percentage	-0.9	-1.8 to 0.05	0.061
Total CSA *	0.6	-0.03 to 1.2	0.062
Contractile CSA #	0.6	-0.02 to 1.2	0.058
Active extension multivariate models for the affected triceps brachii			
Fat percentage	-1.0	-1.8 to 0.1	0.029
Total CSA *	-0.4	-0.2 to 1.0	0.159
Contractile CSA #	0.5	-0.2 to 1.1	0.150
Active supination univariate models for the affected biceps brachii			
Fat percentage	-3.2	-8.0 to 1.7	0.186
Total CSA *	4.8	0.5 to 9.1	0.031
Contractile CSA #	5.0	0.5 to 9.4	0.032
Active supination multivariate models for the affected biceps brachii			
Fat percentage	-3.8	-9.2 to 1.7	0.164
Total CSA *	5.2	0.6 to 9.9	0.030
Contractile CSA #	5.5	0.6 to 10.4	0.030

*The CSA including muscle tissue and intramuscular fat tissue, #contractile CSA = total CSA (100 - mean fat percentage) / 100. Univariate linear regression models evaluated the associate fat percentage, total CSA or contractile CSA of the affected biceps or triceps brachii to active range of motion. The multivariate models were used to correct for age and time after trauma. CI: confidence interval, CSA: cross sectional area.

Table VI: Logistic regression for muscle force in MRC

	Odds ratio	95% CI	*p* - value
Univariate models for the affected biceps brachii			
Fat percentage	-0.17	-0.31 to -0.04	0.012
Total CSA *	0.16	0.02 to 0.29	0.024
Contractile CSA #	0.17	0.03 to 0.32	0.021
Multivariate models for the affected biceps brachii			
Fat percentage	0.7	0.55 to 0.99	0.045
Total CSA *	2.4	1.1 to 5.6	0.034
Contractile CSA #	2.6	1.1 to 6.1	0.029
Univariate models for the affected triceps brachii			
Fat percentage	-0.36	-0.60 to -0.12	0.004
Total CSA *	0.30	0.09 to 0.50	0.005
Contractile CSA #	0.33	0.10 to 0.55	0.005

*The CSA including muscle tissue and intramuscular fat tissue, #contractile CSA = total CSA (100 - mean fat percentage) / 100. Univariate logistic regression models evaluated the contribution of fat percentage, total CSA or contractile CSA of the affected biceps or triceps brachii to the muscle force in MRC 0 to 3 versus MRC 4 and 5. The multivariate models were used to correct for age and time after trauma. Multivariate models of the affected triceps brachii are not shown because of invalid model due zero frequencies. MRC: medical research council, CI: confidence interval, CSA: cross sectional area.

Table VII: Linear regression for muscle force in Newton

Outcome variable	Regression coefficient	95% CI	*p* - value
Univariate models for the affected biceps brachii*			
Fat percentage	-10.3	-15.8 to -4.9	0.001
Total CSA *	12.3	8.5 to 16.1	< 0.001
Contractile CSA #	13.0	9.2 to 16.8	< 0.001
Multivariate models for the affected biceps brachii*			
Fat percentage	-10.3	-16.4 to 4.2	0.003
Total CSA *	12.2	8.1 to 16.3	< 0.001
Contractile CSA #	13.0	8.9 to 17.1	< 0.001
Univariate models for the affected triceps brachii*			
Fat percentage	-15.0	-23.2 to -6.7	0.001
Total CSA *	11.1	6.4 to 15.7	< 0.001
Contractile CSA #	11.7	6.9 to 16.4	< 0.001

Table VII: Linear regression for muscle force in Newton (*continued*)

Outcome variable	Regression coefficient	95% CI	*p* - value
Multivariate models for the affected triceps brachii*			
Fat percentage	-15.4	-24.4 to -6.4	0.002
Total CSA *	12.6	8.3 to 16.9	< 0.001
Contractile CSA #	13.2	8.8 to 17.6	< 0.001
Univariate models for the non-affected biceps brachii			
Fat percentage	-13.4	-41.0 to 14.3	0.323
Total CSA *	7.4	3.6 to 11.2	0.001
Contractile CSA #	7.8	3.8 to 11.8	0.001
Multivariate models for the non-affected biceps brachii			
Fat percentage	-11.9	-40.9 to 17.1	0.396
Total CSA *	7.5	3.5 to 11.5	0.001
Contractile CSA #	7.9	3.6 to 12.1	0.001
Univariate models for the non-affected triceps brachii			
Fat percentage	-1.2	-21.9 to 19.4	0.902
Total CSA *	6.0	-1.0 to 13.0	0.086
Contractile CSA #	6.4	-1.0 to 13.8	0.087
Multivariate models for the non-affected triceps brachii			
Fat percentage	-11.6	-32.2 to 8.9	0.245
Total CSA *	4.1	-3.6 to 11.7	0.270
Contractile CSA #	4.4	-3.4 to 12.3	0.247

*The CSA including muscle tissue and intramuscular fat tissue, #contractile CSA = total CSA (100 - mean fat percentage) / 100. Univariate linear regression models evaluated the contribution of fat percentage, total CSA or contractile CSA of the affected or non-affected biceps or triceps brachii to the muscle force in Newton. *Only n=12 patients were included in these regression models because these patients were able to give muscle force against resistance, i.e. Muscle Research Council (MRC) 4 or 5. The multivariate models were used to correct for age and time after trauma. CI: confidence interval, CSA: cross sectional area.

DISCUSSION

The three-point Dixon MRI quantifies intramuscular fat, total and contractile CSA with an excellent inter-observer reliability in BPI patients. The fat percentage, total and contractile CSA was shown to be homogenous among consecutive MRI slices. The fat percentage of both the biceps and the triceps brachii showed a strong association with the Goutallier score, but the Goutallier score overestimated the fat percentage compared to the Dixon technique. Contractile CSA of the affected biceps brachii contributed most to the reduction in active elbow flexion, active supination and muscle force.

Long-term denervation results in muscle degeneration including muscle atrophy and fatty degeneration. Previous studies in brachial plexus injury used only qualitative methods to score muscular fatty degeneration [4, 9]. Contrary, with quantitative measurement methods comparisons between but also within patient groups during follow-up are more objective. Quantitative MRI has previously been used in patients with rotator cuff tears, in aging and Duchene muscular dystrophy. The values of control patients in literature are comparable with our observation of fat percentage of 6 ± 1.0 % in the non-affected biceps brachii [28, 34]. As hypothetically expected, but never been proved in a clinical setting, contractile CSA was also associated with muscle force of the non-affected biceps brachii indicating a consistent measurement method. Using both quantitative MRI and quantitative muscle force, we calculated the specific muscle force. This excluded the non-functional fat inside the muscle compartment. The specific muscle force was significantly lower in the affected compared to the non-affected biceps brachii, indicating a lower muscle quality in the affected muscle. In BPI, the limited capacity of muscle fibers to contract could be due to the partial denervation, but also muscle stiffness or disorganization of the muscle fibers could influence the capacity of the muscle fibers to generate force [35, 36].

The quantitative three-point Dixon method showed a good correlation with the qualitative T1 measurements of fat using the Goutallier score. As previously described, the Goutallier score gave an overestimation of the intramuscular fat [19]. Other quantitative techniques used to assess fatty degeneration in BPI include ultrasound and computed tomography. However these techniques result in a value for muscle attenuation without the possibility to distinguish between muscle and fat tissue [13, 37]. This is the first study using a quantitative assessment of contractile CSA in BPI patients. Five consecutive MRI slices showed a homogeneous distribution of intramuscular fat, total and contractile CSA. It is not known whether this distribution is also homogeneous along the total length of the muscles as the proximal and distal end of the muscles were not included in the MRI scans in this study.

Strength of this study is the use of the non-affected arm as a control and the association of quantitative MRI data with clinical parameters as range of motion and muscle force. A limitation of this study is a lack of histology; however literature shows an excellent correlation of fat fraction obtained by MRI and histology [38].

Measurements of fat percentage, total and contractile CSA may give more insight into the pathophysiology of contractures and muscle weakness in traumatic BPI as well as neonatal BPI. It could be used to predict which patients are more likely to progress to a worse outcome due to bony deformities and assist at the timing of surgery. Dixon MRI may also improve treatment of BPI by determining which patients favor which type of surgery including contracture releases, tendon

transfers and osteotomies. Measurement of contractile CSA may be used to assess the muscle imbalance around the shoulder in neonatal brachial plexus palsy which causes glenohumeral deformities [4, 10, 12]. Knowledge on this muscle imbalance could assist at decision making on the timing and what kind of operation to perform to prevent glenohumeral deformities. Furthermore, quantitative assessment of fat percentage, total and contractile CSA might be useful in longitudinal follow-up and for research purposes [13]. The contractile CSA of the affected biceps brachii contributed most to the reduction in active flexion, active supination and muscle force. The fat percentage also contributed to clinical outcome, although this contribution was less strong compared to atrophy. As contractile CSA contributed most to clinical outcome, we favor measurement of both the fat percentage and the total CSA to be able to calculate the contractile CSA. Contractile CSA may be the best parameter to quantify muscle atrophy and fatty degeneration, however this will need to be confirmed in future research.

CONCLUSIONS

This study showed that the intramuscular fat, the total and contractile CSA of the biceps and triceps brachii can be assessed in BPI with an excellent reliability. The quantitative scoring of the three-point Dixon sequences was significantly correlated with the qualitative Goutallier score on T1 weighted TSE sequences, however the Goutallier score gave an overestimation. The contractile CSA of the affected biceps contributed most to the reduction in active flexion, active supination and muscle force. Assessment of contractile CSA will yield valuable insight in pathophysiology and predict the outcome of conservative and surgical procedures.

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Chapter 4

Serial casting for elbow flexion contractures in obstetric brachial plexus injury

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ABSTRACT

Background: The objective of this study was to evaluate the effectiveness of serial casting of elbow flexion contractures in obstetric brachial plexus injury.

Methods: A prospective consecutive cohort study was performed with a median follow-up of 5 years. Forty-one patients with elbow flexion contractures ≥30° were treated with serial casting until the contracture was ≤10°, for a maximum of 8 weeks. Range of motion, number of recurrences and patient satisfaction were recorded and analyzed using Wilcoxon signed-rank and Cox regression tests.

Results: Passive extension increased from a median of -40° (IQR -50 to -30) to -15° (IQR -10 to -20, $p < 0.001$). Twenty patients showed 37 recurrences. The baseline severity of passive elbow extension had a hazard ratio of 0.93 (95% CI 0.89 to 0.96, $p < 0.001$) for first recurrence. Median patient satisfaction was moderate. Four patients showed loss of flexion mobility and in two patients serial casting had to be prematurely replaced by night splinting due to complaints.

Conclusion: Serial casting improved elbow flexion contractures, although recurrences were frequent. The severity of elbow flexion contracture is a predictor of recurrence. We recommend more research on muscle degeneration and determinants involved in elbow flexion contractures to improve treatment strategies and prevent side-effects.

INTRODUCTION

Obstetric brachial plexus injury (OBPI) is caused by trauma to the brachial plexus during delivery, resulting in axonotmesis, neurotmesis and/or avulsion of some or all of the C5 through T1 nerve roots and/or trunks. The incidence varies between 0.4 and 4.6 per 1000 live births [1-3]. The natural course is diverse and depends on the extent and severity of the nerve lesions. Most children show full neurological recovery, but 20 to 35% have residual deficits for which reconstructive nerve surgery is indicated [4-8]. Despite improved function after natural recovery and nerve surgery, up to 35% of OBPI patients have residual muscle weakness, contractures and/or joint deformities and require additional treatments to further improve the function of the upper extremity, including soft-tissue releases, muscle tendon transfers and osteotomies [7, 9-11].

The main sequela of upper OBPI (C5-C6) at the elbow joint is flexion contracture, with a prevalence of 50 to 90%. In most cases, flexion contractures are limited to 10° to 30° and can be treated by range-of-motion exercising and nighttime splinting. In a minority of cases, however, the contracture exceeds 30°, and additional treatment is needed. An elbow flexion contracture greater than 30° has a severe impact on function in terms of the international classification of functioning, disability and health (ICF) activity and participation categories, since most daily living activities are executed in 30° to 130° of elbow flexion [12-14]. Literature on the management of elbow flexion contractures in patients with OBPI is scarce, and authors have proposed both non-surgical and surgical treatments, including splinting, serial casting, anterior elbow release and arthrodiatasis [14-19]. However, the studies all had limited patient numbers and lacked long-term follow up. Although serial casting is frequently applied and globally considered to be the preferred therapy, literature on the effect of stretching by serial casting for contractures is limited.

The primary objective of the current study was to evaluate whether serial casting of elbow flexion contractures would improve elbow extension in patients with OBPI. Secondary objectives were to assess the number of recurrences of elbow flexion contractures and to examine side-effects, including the effect of serial casting on biceps and triceps muscle strength and restrictions of flexion, supination and pronation. Patient satisfaction and predisposing patient characteristics influencing treatment outcome were also examined.

METHODS

A prospective consecutive cohort study was performed among children with OBPI treated with serial casting because of elbow flexion contracture.

Patients

Forty-one consecutive patients with OBPI and elbow flexion contracture of ≥30° were recruited at the outpatient clinic of the Leiden University Medical Center (LUMC). All recruited patients were enrolled in this prospective cohort study, as serial casting treatment was the standard treatment protocol for patients with ≥30° of flexion contracture (i.e. an extension deficit of 30°) despite regular physical therapy and nighttime splinting. Serial casting started with two weeks of casting in submaximal stretch to enable the child to get used to the cast and to allow for muscle accommodation. From the third week on, the serial casting was performed in maximal extension to maximize the effect of the casting period. Serial casting was only continued if the treatment was tolerated by the patients. This protocol remained unchanged throughout the inclusion period. Patients were excluded from this study if they had undergone prior surgery of the affected elbow, or if they had radial head dislocation. Patients were also excluded if the elbow flexion contracture benefitted the functional ability of the arm. For example, an elbow contracture can be functional in the case of poor hand function (Raimondi hand function scale scores below 3 [20]). All children and their parents were informed about common discomforts and functional burden during the casting period, like the inability to swim. They were also informed about side-effects like temporary restriction of active elbow flexion after cast removal.

All patients were treated by one staff member (R.G.H.H.N.), and serial casting was performed with weekly casting in maximal elbow extension, until the flexion contracture was 10° or less. Serial casting lasted for a maximum of 8 weeks. If the maximal extension was reached after serial casting, patients received a nighttime splint, using the final cast as a splint. Physical therapy was continued after serial casting, using range of motion exercises. The study protocol to prospectively evaluate children with OBPI was approved by the LUMC ethics committee.

Clinical parameters

The age, gender, affected side, severity of the lesion according to Narakas, type of primary treatment and age at nerve surgery were recorded [21]. All patients were evaluated at baseline, after serial casting (a median of 4 weeks, interquartile range (IQR) 4 to 6) and at annual regular follow-up, including passive elbow extension and flexion as well as passive forearm supination and pronation in 90° of elbow flexion. Elbow flexion and extension strengths were measured on a Medical Research Council (MRC) 0 - 5 scale, as muscle imbalance was considered one of the factors

that can influence recurrence after treatment. Active elbow extension, flexion and forearm supination and pronation were assessed at follow-up to assess side-effects. We also recorded the number of weeks of serial casting, the number of recurrences and whether or not patients received additional treatment. Patient satisfaction regarding goal attainment was retrospectively assessed by an independent pediatric physiatrist, and critically checked by the first author, who subjectively examined the neurosurgeon's, orthopedic surgeon's and rehabilitation records, and rated patient satisfaction using a 5-point Likert scale: 1. very satisfied, 2. moderately satisfied, 3. neither satisfied nor dissatisfied, 4. moderately dissatisfied and 5. very dissatisfied. No patients were lost to follow-up, and baseline and follow-up range of motion data of all patients were available for analysis.

Statistical analysis

The Wilcoxon signed-rank test was used to test the statistical significance of clinical outcome parameters, as range of motion data was not normally distributed. Cox regression analysis was used to determine whether any of the clinical parameters were associated with recurrence of elbow flexion contractures, including age, gender, Narakas type, primary treatment, biceps and triceps muscle strength and number of weeks of serial casting. IBM SPSS Statistics version 20 was used for all statistical testing.

RESULTS

Elbow flexion contracture

Patient characteristics are shown in table I, showing separate data for each Narakas patient category. Clinical results are shown in table II, again showing separate data for each Narakas patient category, including passive extension, flexion, supination and pronation, number of patients with one or more recurrences, mean number of recurrences per patient, time until first recurrence, biceps and triceps muscle strength and patient satisfaction. The elbow range of motion for the total patient group is shown in figure 1. This total patient group had a median passive elbow extension of -40° (IQR -50 to -30) at the start of serial casting. After serial casting for a median of 4 weeks (IQR 4 to 6), the median passive extension had increased to -15° (IQR -10 to -20, $p < 0.001$). At follow-up, after an average of 5 years (IQR 3 to 6), the median passive extension was -25° (IQR -40 to -20, $p < 0.001$). During this period, 20 (49%) patients had a total of 37 recurrences of elbow flexion contracture ≥30° and were treated a second time with serial casting, following the same procedure. The median time to the first recurrence was 2 years (IQR 1 to 4). Excluding patients with recurrent elbow flexion contracture, the median passive extension was increased from -30° (IQR -40 to -30) at baseline to -20° (IQR

-25 to -20, $p < 0.001$) at follow-up. One patient had 7 recurrences of elbow flexion contractures and was treated with multiple serial castings. At an age of 5 years, this patient underwent anterior capsule release because of an elbow flexion contracture of 70°. After 12 years of follow-up, the patient's elbow flexion contracture was 50°. Patient satisfaction was scored at a median value of 2, indicating 'moderately satisfied' (IQR 1 `very satisfied´ to 2 ´moderately satisfied´).

Side-effects / complications: elbow flexion, supination and pronation

Two participants scored 5 on the satisfaction scale, indicating 'very dissatisfied' as regards goal attainment, in both cases due to loss of elbow flexion. Two participants reported greater than expected discomfort in the first week of serial casting, including pain and hand edema. In both of these children, the serial casting had to be prematurely replaced by splinting as a result of complaints. The median baseline passive elbow flexion was 150° (IQR 145 to 150). After serial casting for

Table I: Patient characteristics

Narakas type	C5 – C6 N = 11	C5 – C7 N = 21	C5 – T1 N = 9
Sex (male)	5	8	6
Age at baseline measurement # * (years)	6.8 (4.71)	7.1 (4.36)	10.9 (4.68)
Side (right / left)	7 / 4	10 / 11	4 / 5
Age at primary treatment § * (years)	0.5 (0.26)	0.5 (0.17)	0.4 (0.24)
Conservative / neurolysis / nerve transplantation	8 / 1 / 2	6 / 1 / 14	1 / 0 / 8
Anterior division of superior trunk	2	10	8
Posterior division of superior trunk	2	9	7
Medial trunk	1	1	5
Suprascapular nerve	2	9	6
Secondary procedures			
Shoulder external rotation surgery	3	9	3
Pronation osteotomy of the ulna and/or radius	0	4	2
Wrist extension surgery	0	4	3
Botulinum toxin injection subscapularis	1	0	0
Botulinum toxin injection triceps	0	0	1
Botulinum toxin injection biceps and brachialis †	0	1	0
Biceps rerouting surgery †	0	2	1
Elbow manipulation in narcosis †	1	0	0
Anterior capsule release elbow †	1	0	0
Casting ‡ (weeks)	6 (3 to 7)	4 (3 to 5)	6 (4 to 6)
Follow-up * (years)	5.4 (4.15)	5.6 (3.03)	3.0 (2.97)

#This is the age at the start of serial casting treatment. *The values are given as mean with standard deviation. §This is the age at neurosurgical intervention. †Secondary procedure performed after inclusion. ‡The values are given as median and interquartile range.

a median of 4 weeks (IQR 4 to 6), the median passive flexion had decreased to 140° (IQR 120 to 150, $p = 0.001$) and at a mean follow-up of 5.0 years (SD 3.29) it had increased again to 145° (IQR 140 to 150, $p = 0.006$) as shown in figure 1. Four patients showed a loss of elbow flexion of 50° or more. One child with a severe decrease in elbow flexion, to 85°, had to be treated two times with manipulation under general anesthesia, after which the elbow flexion stabilized at 130°. During follow-up, six patients were treated with pronation osteotomy of the ulna and/or radius, including three patients who were additionally treated with biceps tendon transfer at a mean of 5.3 years (SD 1.05) after the start of serial casting. However, these supination contractures had no relation with the serial casting. When these six patients are excluded, there was no change in the median passive supination at follow-up ($p = 0.13$). The median baseline passive pronation was 90° (IQR 30 to 90) and had not changed either after serial casting or at the final follow-up. The median

Table II: Results of serial casting treatment

Narakas type	C5 – C6 N = 11	C5 – C7 N = 21	C5 – T1 N = 9
Passive extension ‡ (°)			
At baseline	-30 (-40 to -30)	-40 (-48 to -30)	-45 (-50 to -38)
After 4 weeks	-20 (-20 to -14)	-15 (-20 to -10)	-13 (-20 to -6)
At follow-up	-25 (-30 to -20)	-25 (-40 to -20)	-30 (-40 to -18)
Passive flexion ‡ (°)			
At baseline	150 (145 to 150)	150 (145 to 150)	150 (143 to 150)
After 4 weeks	143 (101 to 150)	140 (120 to 150)	140 (100 to 150)
At follow-up	143 (130 to 150)	145 (140 to 150)	145 (140 to 150)
Passive supination ‡ (°)			
At baseline	90 (88 to 90)	90 (74 to 90)	90 (85 to 100)
After 4 weeks	90 (90 to 90)	90 (90 to 90)	90 (90 to 96)
At follow-up	90 (78 to 90)	70 (30 to 90)	90 (10 to 90)
Passive pronation ‡ (°)			
At baseline	90 (80 to 90)	90 (38 to 90)	30 (5 to 90)
After 4 weeks	90 (90 to 90)	65 (39 to 83)	10 (-11 to 90)
At follow-up	90 (80 to 90)	80 (60 to 90)	70 (0 to 90)
Patients with recurrence	4 (36%)	10 (48%)	6 (67%)
Recurrences per patient #*	3 (3.0)	2 (1.0)	2 (0.8)
Time of first recurrence * (years)	2.9 (2.02)	2.2 (1.85)	2.3 (1.08)
Biceps muscle strength ‡ (MRC)	4 (4 to 4)	4 (4 to 5)	4 (3 to 4)
Triceps muscle strength ‡ (MRC)	5 (5 to 5)	4 (3 to 5)	4 (3 to 4)
Patient satisfaction ‡	2 (1 to 3)	2 (1 to 2)	2 (2 to 3)

‡The values are given as median and interquartile range. # The mean number of recurrences of elbow flexion contracture per patient after the initial serial casting treatment. *The values are given as mean with standard deviation.

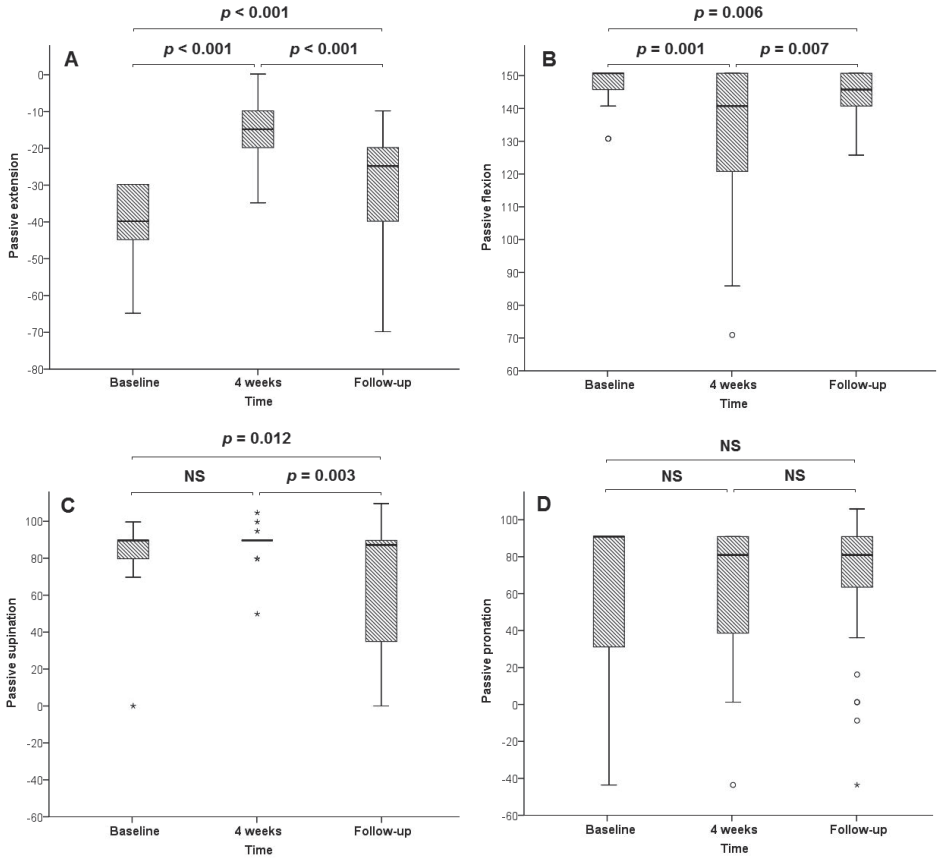

Figure 1: Clinical results
Clinical results presented as box plots of passive extension (A), flexion (B), supination (C) and pronation (D) at baseline, after a median of 4 weeks of serial casting and at final follow-up after 4.6 years. Wilcoxon signed-rank *p*-values are shown for changes between baseline measurement, measurement after a median of 4 weeks of serial casting, and measurements at follow-up. NS = not significant.

biceps muscle strength in the total patient group was MRC 4 (IQR 4 to 4) and the median triceps muscle strength was MRC 4 (IQR 3 to 5), both of which did not change during follow-up. As expected, the median triceps muscle strength was lower in patients with C7 involvement (MRC 4, IQR 3 to 5) than in patients with a C5-C6 lesion (MRC 5, IQR 5 to 5, $p = 0.02$). The median biceps muscle strength (MRC 4, IQR 4 to 4) was lower than the median triceps muscle strength (5, IQR 5 to 5) in patients with a C5-C6 lesion ($p = 0.025$). Biceps and triceps muscle strengths were comparable in patients with a C7 involvement ($p = 0.34$). The median passive elbow extension at baseline was lower in patients with C7 involvement (-40°, IQR

-50 to -30) than in those with a C5-C6 lesion (-30°, IQR -40 to -30). This difference approached significance ($p = 0.07$). At follow-up, the median active extension was -30° (IQR -40 to -20), median active flexion 140° (IQR 135 to 145), median active supination 70° (IQR 10 to 90) and median active pronation 80° (IQR 40 to 85).

Predictors of recurrent elbow flexion contractures

Baseline median passive extension was -45° (IQR -60 to -30) in patients with recurrence of elbow flexion contracture, compared to -30° (IQR -40 to -30) in patients without recurrence, but this difference did not reach statistical significance ($p = 0.16$). Patients whose flexion contracture recurred showed no significant difference in passive flexion, supination or pronation at baseline or after serial casting, compared to patients without recurrences. However, their median extension decreased further during follow-up, despite further serial casting treatments, from -20° (IQR -20 to -10) to -40° (IQR -50 to -30, $p = 0.001$). At follow-up, patients with and without recurrence of flexion contracture did not differ in terms of passive or active flexion, supination or pronation, or biceps or triceps muscle strength.

Multivariate cox regression was used to model the time to first recurrence of elbow flexion contracture. Severity of passive elbow extension at baseline had a hazard ratio of 0.93 (95% CI 0.89 to 0.96, $p < 0.001$) for first recurrence of elbow flexion contracture. The following factors were not associated with recurrence of elbow flexion contracture: age, gender, Narakas type, primary treatment, biceps or triceps muscle strength and number of weeks of serial casting.

DISCUSSION

In the present study, serial casting of elbow flexion contractures in children with OBPI improved passive elbow extension, although recurrence of elbow flexion contractures was frequently observed (49%). Passive elbow flexion was decreased after serial casting. Four cases of a severe decrease in elbow flexion (i.e. more than 50° loss of elbow flexion) were encountered after casting, for which one patient required manipulation under general anesthesia. Although all patients in this population recovered during follow-up, the severe decrease in elbow flexion should be interpreted as a serious side effect. Passive supination and pronation were not affected by serial casting, and no changes were observed in biceps or triceps muscle strength. The more severe the contracture was at the start of the serial casting, the higher the likelihood of recurrence.

In terms of the ICF activity and participation levels, elbow flexion contractures are generally associated with functional hand positioning limitations, for example during balancing, cycling, or activities like carrying a bag or leaning on a bar. Literature on functional issues is scarce, and many questions remain about

the clinical relevance of treating elbow flexion contractures. A study by Morrey demonstrated that most daily living activities are executed in the 30° to 130° range of motion of elbow flexion [13], and skills such as carrying a bag require even more elbow extension. These arguments prompted us to treat patients with persistent elbow flexion contractures ≥30° with serial casting. Treatment goals in our study also related to cosmetic issues. Patients with elbow flexion contractures below 30° are generally treated with physical therapy and night splinting only.

Literature on the treatment of elbow flexion contractures ≥30° is limited. In a recent study by Sheffler et al., 9 patients with elbow flexion contractures ≥30° were treated with serial casting [14]. They showed an improvement in passive elbow extension from -49° to -30°, which is less favorable than the results in the present study, which found an improvement in passive elbow extension from -40° to -15°. The more favorable outcome in our population (passive elbow extension -15° versus -30°) could be the result of an earlier start of the serial casting and the duration of serial casting, which was not reported by Sheffler et al. [14]. These authors found a further deterioration of the elbow flexion contracture of 4.4% a year during 27 months of follow-up, which is consistent with our finding of 10° of deterioration over 5 years of follow-up. Ho et al. treated 19 patients non-surgically for elbow flexion contractures, and found an improvement in passive elbow extension from -48° to -17°. However, their results included patients treated with serial casting as well as those treated with splinting [17].

The etiology of flexion contracture of the elbow in children with OBPI is unknown and needs further elucidation [10]. Current hypotheses in the literature include reinnervation of the elbow flexors prior to that of the elbow extensors, leading to muscle imbalance, and/or co-contraction as a cause of elbow flexion contractures [12]. Our study found no differences in biceps and triceps muscle strength of patients with lesions of both C5-C6 and C7. Patients with lesions only of C5-C6 developed an elbow flexion contracture despite stronger triceps than biceps muscles. Thus, the results of our study do not support muscle strength imbalance as the cause of elbow flexion contractures. Other hypotheses include changes in the partially denervated muscle itself as a cause of elbow flexion contracture, including muscle atrophy, fattening and fibrosis [22]. It is also possible that elbow flexion contractures could be caused by positional preferences of the arm in brachial plexus injury. A mild elbow flexion contracture could facilitate the weak elbow flexors, as limitation of elbow flexion leads to greater impairment of daily activities [13]. This, in conjunction with the internal rotation contracture present in the shoulder, attenuates the flexion position of the elbow even further.

Strengths of this study include that it is the largest case series so far on the treatment outcome of OBPI patients with severe elbow flexion contractures. We present clinical outcomes and complication rates, giving further clues about the etiology of elbow flexion contractures. To our knowledge there have not been any

reports of serial casting studies using a 5-year follow-up. A limitation of this study is the lack of control groups to compare different treatment modalities, making it a prospective consecutive cohort study. The global use of the therapy and its presumed effect make it difficult to obtain ethical approval for including control groups and to motivate parents to participate in a randomized controlled trial. One of our messages is that the effect should not be overestimated, and we strongly recommend that randomized controlled trials be organized. Another limitation is that therapy compliance regarding night splinting and co-interventions like physical therapy were not recorded, which might have influenced recurrences of elbow flexion contracture. A third limitation concerns a lack of reproducibility as a result of individual variation in therapy. The maximum stretch force applied during serial casting depends on the interaction with and tolerance of the child and/or parent and therefore might have influenced the outcome of the serial casting treatment. On the other hand, a strength of this study is that the intervention performed was usual care, and therapy did not change because of participation in the study. This was also the reason why we had not expected the dissatisfaction that we found, and we therefore had to assess patient satisfaction retrospectively. Future studies should assess the patient satisfaction and goal attainment prospectively.

In conclusion, this study demonstrates that serial casting can improve elbow flexion contractures in children with OBPI. Complications include temporary decrease of elbow flexion and a high rate of recurrence of elbow flexion contractures, stressing the importance of close follow-up. The severity of the elbow flexion contracture at the start of serial casting is a predictor of recurrence of the elbow flexion contracture. Further research into the etiology and treatment strategies could optimize the management of elbow flexion contractures in OBPI. This could include studies on the many recent developments in dynamic splinting techniques. As including an untreated control group is hardly feasible, we recommend randomized controlled trials comparing generally accepted therapy strategies such as serial casting and dynamic splinting. More research is needed to evaluate serial casting of elbow flexion contractures at the ICF level of activity and participation. In order to prevent side-effects, we recommend a close follow-up, careful physical examination during the cast changes and critical estimation of the casting period, which should be as short as possible.

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Chapter 5

Botulinum toxin injection for internal rotation contractures in obstetric brachial plexus injury A minimum 5-year prospective observational study

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ABSTRACT

Background: Obstetric brachial plexus injury (OBPI) is frequently associated with internal rotation contractures of the shoulder as a result of muscle imbalance. The purpose of this study is to assess the effect of botulinum toxin A (BTX-A) injection in the subscapular muscle on external rotation and the need for tendon transfer for external rotation of the shoulder.

Methods: A prospective comparative study was performed including 15 consecutive patients treated with BTX-A and a historic control group of 67 patients with mean age 30 months (SD 10). The BTX-A injection (2 IU/kg body weight) was performed immediately following MRI under general anesthesia in the subscapular muscle. Passive external rotation, the need for tendon transfer surgery, glenohumeral deformity and muscle degeneration were evaluated. The hazard ratio for no relapse of internal rotation contracture after BTX-A injection compared to no BTX-A injection was calculated.

Results: In the BTX-A group, the passive external rotation in adduction increased from -1° (95% CI -10 to 8) to 32° (95% CI 17 to 46) at 3 months and 6 patients were indicated for surgery compared to a decline from -2° (95% CI -7 to 3) to -11° (95% CI -17 to -6) in the control group with 66 indications for surgery. At 5 years follow-up, 10 patients in the BTX-A group were indicated for surgery with a hazard ratio of 4.0 (95% CI 1.9 to 8.4).

Conclusions: BTX-A injection in the subscapular muscle of OBPI patients can reduce internal rotation contractures and subsequently the need for tendon transfer surgery. At 5 years follow-up a relapse was seen in 67% of the patients treated with BTX-A. Since at MRI less SC degeneration was found in the good responders on BTX-A treatment, this group seems to be the best target group. Further research is needed on patient selection for BTX-A injection including glenohumeral deformity, subscapular degeneration as well as doses of BTX-A to be used.

Level of Evidence: Level II - prospective comparative study.

INTRODUCTION

Obstetric brachial plexus injury (OBPI) patients often develop internal rotation contractures with a prevalence of up to 39% depending on the extent and severity of the brachial plexus injury [1, 2]. Several theories on the origin of an internal rotation contracture in OBPI patients exist. The muscle imbalance theory states that OBPI leads to muscle imbalances around the shoulder, in which internal rotators are stronger resulting in an internal rotation contracture [3, 4]. But also posture by which the injured extremity is held close to the body to enable easier bimanual activities, will cause a contracture if this position is unopposed by active external rotation. Recently an animal study has shown that selective denervation of the subscapular muscle (SC) alone leads to SC atrophy and internal rotation contracture indicating that weakness of the external rotators are not solely responsible for the muscle imbalance causing internal rotation contracture [5]. Furthermore, excision of the external rotators in mice without brachial plexus injury caused no contractures or shortening of the SC muscle [6]. Previously, MRI studies have shown that upon brachial plexus injury the muscle degeneration was most prominent in the SC muscle [7].

Treatments of internal rotation contractures include surgical SC release. These techniques are combined with transfer of a latissimus dorsi and/or teres major tendon to the rotator cuff to create active external rotation to improve arm function and quality of life [8-10]. Disadvantages of SC release and/or tendon transfer include weaker adduction and potential partial power loss of internal rotation with a subsequent risk for an external rotation contracture of the shoulder. A less invasive method to address the internal rotation contracture is the injection of botulinum toxin A (BTX-A) [10-12]. There have been some reports on BTX-A injections but no clear conclusions can be drawn from these studies since the heterogeneity was large (ie number of BTX-A injections, variety of muscles, combination with tendon transfer surgery) [13-18].

Our hypothesis is that injection of BTX-A into the SC muscle alone could temporarily weaken its function to open a time window primarily for the treatment of the internal rotation contracture with intensive physical therapy, but also to give the external rotation movement time to get "learned" (i.e. cerebral plasticity) again during global movement of the upper extremity. There have been no reports on BTX-A injection in the SC to treat internal rotation contracture of OBPI children without tendon transfer surgery.

The primary objective of the present study was to assess the efficacy of BTX-A injection in the SC on the passive external rotation (PER) in children with OBPI. Because of a potential increase of PER, the need of tendon transfer could decrease after BTX-A treatment. Therefore the second objective was to assess the effect of BTX-A injection on the number of indications for tendon transfer surgery. The

third objective was to investigate whether patient or MRI characteristics influence BTX-A treatment.

METHODS

Patients

A prospective comparative study was performed with at least five year follow-up on 15 OBPI patients with an internal rotation contracture treated with BTX-A in the SC muscle. Clinical outcome of these BTX-A OBPI patients were compared to a historic prospective control group of 67 patients with an internal rotation contracture. BTX-A was used after written informed consent was obtained from the parents.

Between 1997 and 2009 all patients with OBPI were seen at the outpatient clinic of the Leiden University Medical Center. Only those patients with a progressive internal rotation contracture were included in the present study, regardless whether the OBPI lesion was initially treated conservatively or by nerve surgery. From 2007 onwards, all patients younger than 48 months old were injected with 2 IU/kg BTX-A (Botox ®, Allergan Inc.) in the two parts of the SC muscle. Patients treated before 2007 were used as a historical control group. Management of all patients, both the historical control group and the BTX-A group, consisted of daily stretching exercises supervised by a trained physical therapist for at least 3 months. A progressive internal rotation contracture was defined as an external rotation in adduction of less than 30 degrees. In all patients, the passive external rotation (PER) range of motion was reduced to 30° or less, and the Mallet functional shoulder score was 3 or less for the subsets hand-to-mouth and/or hand-to-head movement [19]. A standardized MRI of the shoulder was performed under anesthesia, which is part of the preoperative work-up for children eligible for an external rotation tendon transfer in our clinic [7]. In the control group, all patients eligible for MRI were considered to have a surgical contracture release and a tendon transfer after the MRI. The time elapsed between MRI and surgery depended on the surgical waiting list.

To reduce any potential sources of bias, consecutive patients were included. Furthermore, in both the historical patient group and the BTX-A group, patients were excluded if a complete posterior dislocation of the humeral head was present at MRI. A complete dislocation was defined as a smaller than 10% part of the humeral head being anterior to the longitudinal axis of the scapula (PHHA) [20]. These dislocated shoulders were considered to be beyond the point of a correctable joint. Patients with prior secondary orthopedic surgery or Raimondi hand function scale less than 3 were excluded as well [21].

The affected side, severity of the lesion according to Narakas, type of primary treatment and age at nerve surgery was recorded [22]. All patients were evaluated at 3 months and yearly after the BTX-A injection at the outpatient clinic. All patients completed 5 year follow-up and were included in the data analysis. If a tendon transfer was not indicated, then patients were scheduled for clinical follow-up. The medical ethical review board of the Leiden University Medical Center approved for the prospective database of orthopedic interventions of OBPI patients.

Clinical assessment

The PER of the glenohumeral joint was assessed in adduction and 90° abduction using a hand held goniometer. The passive external rotation range of motion was measured with the elbow flexed to 90° and with the hand of the examiner holding the scapula (i.e. the acromion). True glenohumeral external rotation range was measured at the position where the first sign of resistance (i.e. movement of scapula with respect to the humeral bone) while external rotating the arm was felt. No force was exerted on the arm in order to avoid a shift of the scapula which introduces a thoracoscapular component in the total external rotation range. Negative degrees denote internal rotation from neutral position. Furthermore, the passive glenohumeral abduction was measured and the passive internal rotation was measured in 90° abduction. The Mallet score was used to assess global active shoulder function [19].

BTX-A injection

The BTX-A injection was performed immediately following the MRI under general anesthesia in fifteen patients. In one patient the BTX-A injection was performed two months after the MRI was performed under general anesthesia. Patients were put in the lateral decubital position with the affected arm in maximum internal rotation and adduction to reach winging of the medial edge of the scapula. A flacon of 100 IU BTX-A was diluted in 10 ml 0.9% NaCl. In total, 2 IU per kg was injected per patient. A nine cm 22 gauge slightly bowed needle was inserted anterior of the medial scapular edge at one- and at two-thirds of the distance between the angulus superior and inferior of the scapula to block the motor endplates of the upper subscapular and the lower subscapular nerves. When the needle touched the scapular bone, the needle was retracted a few millimeters to ensure that the BTX-A was injected in the SC [23]. Slightly bowing the needle did never result in breaking. Furthermore, neurovascular injury or pneumothorax did not occur. After BTX-A injection parents were instructed to continue the daily stretching exercises just as before the BTX-A injection supervised by a trained physical therapist. Throughout the study there were no changes in the BTX-A injection or physical therapy instructions.

MRI

The MRI images were acquired using a 1.5 Tesla magnet (Philips Healthcare Inc.). T1 images were made in the transverse plane of the shoulder. For all sequences, the slice thickness was 4.0 mm with a 0.4 mm spacing gap. The degree of glenoid version and PHHA were measured in a transverse plane of the shoulder at midglenoid level, as previously described [7, 20, 24]. The degree of SC degeneration was measured on a 3-point visual scale. The SC was graded as normal if the diameter of the SC of the affected and the contra lateral shoulder were similar. The SC was graded as atrophic if the diameter was smaller. If fatty streaks were also present, the SC was graded as atrophic with fatty degeneration [7]. To measure the interobserver variability, 2 independent observers evaluated the PHHA and glenoid version of 15 patients (R.G.H.H.N. and B.J.D.) and the SC degeneration of 50 patients (S.H. and B.J.D.). One investigator (B.J.D.) repeated the scoring at an interval of two weeks to measure the intraobserver variability. The interobserver variability of the MRI variables was excelled for glenoid version (ICC 0.87), PHHA (ICC 0.96) and SC degeneration (kappa 0.77) as was the intraobserver variability for glenoid version (ICC 0.92), PHHA (ICC 0.96) and SC degeneration (kappa 0.79).

Statistical analysis

Statistical differences were tested by the Pearson's Chi-Square test for nominal categorical variables, the Fisher's exact test for nominal categorical variables if more than 20% of the cells had an expected value of less than 5 and the Mann-Whitney test was used for ordinal categorical variables. The Student independent sample t-test was used for continuous variables with 95% confidence intervals (CI). Differences in Mallet scores were tested using the Wilcoxon Signed Ranks test. Kaplan Meyer analysis was used to calculate survival probability of conservative therapy with 95% confidence interval of the BTX-A patient group. The interclass correlation coefficient (ICC) was calculated for reliability testing of the PHHA and glenoid version, using the 2-way random model with absolute agreement [25]. The linear weighted kappa was calculated for reliability testing of SC degeneration [26]. For interpretation, the criteria formulated by Cichetti and Sparrow were used: 0.00 to 0.39, poor; 0.40 to 0.59, fair; 0.60 to 0.74, good; or 0.75 to 1.00, excellent [27]. For statistical analysis a SPSS software package was used (SPSS Inc., version 20.0, Chicago, Illinois). For the Kaplan Meier analysis R was used (The R foundation for statistical computing, version 3.1.2, Austria). All analyses were two tailed and *p*-values < 0.05 were considered significant.

RESULTS

Range of motion

The individual characteristics of patients treated with BTX-A and the control group are summarized in Table I. No significant differences were found between gender, age, affected side, Narakas type and type of primary treatment. No adverse events were observed following BTX-A injection. The results of the PER and indications for tendon transfer surgery for both groups are summarized in Table II. At baseline, the mean PER in adduction was -1° (95% CI -10 to 8) in the BTX-A group and -2° (95% CI -7 to 3) in the control group. In the BTX-A group, the mean PER in adduction was increased to 32° (95% CI 17 to 46) after 3 months follow-up. In the control group, who eventually had surgery, the follow-up time was determined by the waiting list for surgery. All patients were assessed at the day before surgery again. The mean follow-up time of the control group was 5.7 (SD 2.2) months. These patients showed a further decline with a mean PER in adduction to -11° (95% CI -17 to -6).

The PER in abduction in the BTX-A group increased from 55° (95% CI 45 to 65) to 65° (95% CI 55 to 80, $p = 0.014$) after three months. No significant changes were observed after 1 or 5 years. The median passive abduction was 90 degrees (interquartile range 90-90) and did not change during follow-up. The passive internal rotation in abduction increased from 45° (95% CI 35 to 55) to 65° (95% CI 50 to 85, $p = 0.005$) after 5 years follow-up. The Mallet score did not significantly change for the BTX-A or the control group at follow-up.

Tendon transfer surgery

At follow-up after the BTX-A injections, patients were indicated for tendon transfer surgery if the internal rotation contracture persisted (PER in adduction 30° or less) in presence of no active external rotation, both indicating a Mallet functional shoulder score of 3 or less for the subsets hand-to-mouth and/or hand-to-head movement. Survival probability of conservative therapy is shown in the Kaplan Meier curve of figure 1. In the BTX-A group, six patients (40%) were indicated for tendon transfer after 3 months. Nine patients showed an improvement in PER in adduction and were therefore not indicated for tendon transfer surgery. In contrast, only one patient in the control group showed (spontaneous) good clinical function at follow-up and 66 patients (99%) were indicated for tendon transfer surgery. At 5 years follow-up, 10 patients (67%) in the BTX-A group showed an internal rotation contracture relapse and were therefore indicated for tendon transfer surgery. The hazard ratio for no relapse after BTX-A injection compared to no BTX-A injection was 4.0 (95% CI 1.9 – 8.4).

Table I Baseline characteristics

Number	Age*	Sex	Side	Narakas type	Primary treatment	Glenoid version†	PHHA	SC score#
BTX-A Group								
1	12	Female	Right	C5-C6	Conservative	-21	48	3
2	14	Male	Left	C5-C7	Nerve surgery	-28	17	3
3	16	Male	Right	C5-T1	Nerve surgery	-16	44	2
4	17	Male	Left	C5-C6	Conservative	-21	37	1
5	18	Male	Left	C5-C7	Nerve surgery	-38	40	3
6	21	Male	Left	C5-C6	Nerve surgery	-28	38	2
7	23	Male	Right	C5-C6	Conservative	-30	44	2
8	24	Male	Left	C5-C7	Nerve surgery	-29	16	3
9	41	Male	Left	C5-C6	Conservative	-22	39	1
10	42	Female	Left	C5-C6	Nerve surgery	-14	42	3
11	42	Male	Right	C5-C6	Neurolysis	-21	38	3
12	43	Male	Left	C5-C7	Nerve surgery	-9	46	3
13	44	Female	Right	C5-C6	Nerve surgery	-17	44	3
14	47	Female	Left	C5-C7	Nerve surgery	-18	39	3
15	51	Female	Right	C5-C6	Nerve surgery	-12	46	3
Control Group								
67 patients	30 (8.7)‡	30 male	40 right	34 C5-C6 27 C5-C7 1 C5-C8 5 C5-T1	14 conservative 3 neurolysis 50 nerve surgery	-21 (7.9)‡	32 (10.7)‡	5 score 1 15 score 2 47 score 3

*Age is given in months. † Glenoid version is given in degrees. # SC = subscapularis muscle was scored as 1: normal, 2: atrophic or 3: atrophic with fatty degeneration. ‡ The values are given as mean and standard deviation. MRI = magnetic resonance imaging, BTX-A = botulinum toxin A, PHHA = the percentage of the humeral head anterior to the transverse axis of the scapula.

Table II Clinical outcome of BTX-A injection vs. control group

Number	Passive external rotation in adduction (degrees)				Mallet score at final follow-up						Tendon transfer surgery at final follow-up (months)*
	Baseline	3 months	1 year	5 years	Abduction	External rotation	Hand - head	Hand - back	Hand - mouth	Aggregate	
BTX-A Group											
1	-10	60	25		4	1	4	4	2	15	36
2	-10	-15									3
3	30	65	20	40	4	3	4	4	3	18	None
4	0	40	40	25	4	2	4	4	4	18	None
5	0	10									3
6	10	50	30		4	1	4	4	3	16	36
7	10	20			4	1	3	3	3	14	3
8	-10	0			3	1	2	2	3	11	3
9	0	80	30	20	4	2	4	4	4	18	None
10	-40	40	-20		4	1	3	4	3	15	12
11	0	15			4	1	4	3	3	15	3
12	-10	20	0	10	3	1	3	3	3	13	None
13	15	25			4	1	3	2	3	13	3
14	0	45	35	25	4	1	4	2	3	14	None
15	0	20	0		2	1	2	3	2	10	24
Control group											
67 patients	0 (20)#	-10 (20)#			3 (1-4) †	3 (1-4) †	3 (1-4) †	3 (1-4) †	3 (1-4) †	3 (1-4) †	66 patients

Clinical outcome of the BTX-A group and the control group as baseline and follow-up. In the control group the mean follow-up time was 6 months which was the time elapsed between MRI and surgery depended on the surgical waiting. *Patients with relapse of internal rotation contracture were indicated for tendon transfer surgery. #Values are given as mean with standard deviation. †Values are given as median with range. BTX-A = botulinum toxin A.

MRI and patient characteristics

MRI characteristics of the BTX-A and the control group are shown in Table I. The glenoid version, PHHA and SC degeneration were not different between the groups. Of the 5 patients in the BTX-A group with still a good response after 5 years follow-up, the SC score was normal in 2 patients (40%), atrophic in 1 patient (20%) and atrophic with fatty degeneration in 2 patients (40%). Whereas in the 10 patients who were indicated for tendon transfer surgery, the SC score was normal in none of the patients, atrophic in 2 patients (20%) and atrophic with fatty degeneration in 8 patients (80%), however this was not significantly different (p = 0.08). No significant differences were found in age, gender, Narakas type, primary treatment, baseline PER, glenoid version or PHHA between the good responder group and the patients indicated for tendon transfer surgery.

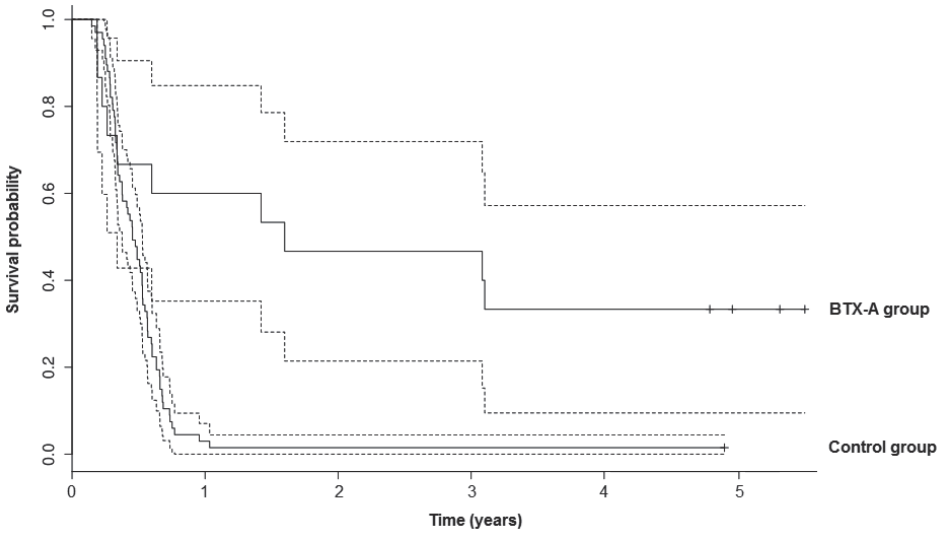

Figure 1: Survival after BTX-A treatment versus control group
Kaplan Meyer curve with survival probability of conservative therapy with 95 % confidence interval of the botulinum toxin A (BTX-A) and control group. Tendon transfer surgery was indicated if at follow-up the passive external rotation (PER) range of motion persisted at 30° or less and the Mallet functional shoulder score was 3 or less for the subsets hand-to-mouth and/or hand-to-head movement.

DISCUSSION

The purpose of the present study was to assess the efficacy of BTX-A injection in the SC to improve the PER in children with OBPI. No adverse events were observed, therefore BTX-A injection can be considered to be safe and feasible in this patient group with a mean age of 2.5 years old. The results of this study show that BTX-A injection increases PER in adduction compared to the control group. Addressing internal rotation contracture of the shoulder is important since progressive glenohumeral joint deformity occurs after persisting internal rotation contracture in OBPI [7, 20]. The reason to focus on the SC muscle to treat the contracture was that the SC muscle is the main constrained in adduction of the arm and source of internal rotation contracture. The results of this study are clinically relevant since the improvement of PER was sufficient to postpone tendon transfer surgery for at least five years and prevent tendon transfer surgery in 33% of patients in the BTX-A group. These good responders on BTX-A injection showed less SC muscle degeneration. This difference did not reach statistical significance, most probably due to the low number of patients treated. Lack of effect on BTX-A injection in degenerated muscle (fibrosis or fatty degeneration) could be a result of absence of a target for the BTX-A, since no or little muscle fibers are present.

In previous studies many different muscles were injected (pectoralis major and minor, teres major, SC and latissimus dorsi), in a variety of number of injections (1 to 4) and patients with a variety of ages (range 0.3 to 13.5 years old) [13-17]. In a recent study Michaud et al. found a mean increase of 6° in PER after BTX-A injections in the muscles which altered the surgical plan in 4 of the 18 patients, however also multiple muscles were injected (pectoralis major, SC and / or latissimus dorsi) in patients with variable ages (range 0.5 to 10 years) with short follow-up of 1 year [18]. The present study is the first controlled study on BTX-A injections in the SC of OBPI patients to correct internal rotation contractures.

In this study the improved clinical effect outlasted the therapeutic time window of BTX-A. This phenomenon could be explained by the time window opened by the BTX-A injection which causes relaxation of the SC muscle during which physical therapy could be more effective. Furthermore, relaxation of the SC muscle reduce afferent signals to the brain and gives time for cortical recruitment for the injured nerves leading to an altered balance between afferent input and motor output [15, 28].

Limitations of this study include the lack of long term (beyond five years) effects on PER and the need for tendon transfer surgery in the future remain unknown at this moment. Because glenohumeral joint morphology and SC degeneration affect shoulder functional outcome, we measured the PHHA, glenoid version and the SC degeneration of the affected and normal shoulder on MRI. As previously observed, we found a significant difference in glenoid version and PHHA of the affected

shoulder compared to the normal shoulder [7, 20]. This study excluded patients with severe glenohumeral deformity and/or complete posterior dislocation of the humeral head, since it was considered that this deformity was beyond a passively correctable joint. Finally, this study is a non-randomized study, thus confounders might be present for example the willingness of the parents to practice the external rotation.

In conclusion, this prospective observational study demonstrates that BTX-A injections in the SC of OBPI children with an internal rotation contracture reduces the internal rotation contracture and could potentially postpone and in some cases prevent external rotation tendon transfer surgery. The beneficial effect of BTX-A injection due to relaxation of the SC muscle opens a time window for both intensive exercises of external rotation, as well as teaching the child to make this movement part of its global movement of the extremity (i.e. cerebral plasticity). Both pathways will reduce the internal rotation contracture resulting in a new balance between the external and internal rotators of the shoulder. This is however not valid in all patients since at minimum 5 years follow-up a relapse of internal rotation contracture was seen in 67% of the patients treated with BTX-A. Since at MRI less SC degeneration was found in the good responders on BTX-A treatment, this group seems to be the best target group. BTX-A injection in multiple injection sites of the subcapular muscle could optimize the effectiveness of BTX-A treatment as anatomic studies showed variability of the subscapular innervations [29, 30]. This study focused on patients of 4 years old or younger as young patients may have more reinnervation potential and more cerebral plasticity to strengthen the external rotators. Future research could focus on patients older than 4 years to investigate whether or not the indication for BTX-A treatment could be extended to older patients with OBPI. Further research is needed on patient selection for BTX-A injection including glenohumeral deformity, SC degeneration as well as doses of BTX-A to be used and whether repeating the BTX-A injection could further reduce the internal rotation contracture.

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Chapter 6

Muscle characteristics in patients with chronic systemic inflammation

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ABSTRACT

Introduction: Histological characteristics of age-related muscle wasting are type II muscle fiber atrophy, accumulation of oxidative stress-induced lipofuscin granules and decreased satellite cell numbers. There is increasing clinical evidence for a strong correlation between chronic systemic inflammation and age-related muscle wasting. The aim of this study was to determine the impact of chronic systemic inflammation on age-related histological muscle characteristics.

Methods: As a model for chronic systemic inflammation, we included 10 patients suffering from rheumatoid arthritis (RA) and 27 control patients suffering from osteoarthritis (OA). Biopsies were taken from the vastus medialis muscle.

Results: No significant differences were found in type II muscle fiber atrophy, lipofuscin accumulation, or satellite cell number in RA compared with OA patients.

Conclusions: These results suggest there is no association between chronic systemic inflammation in RA and age-related muscle characteristics. Future research should focus on inflammation and satellite cell function.

INTRODUCTION

Age-related loss of skeletal muscle mass (sarcopenia) is a major contributor to disability and mortality [1-3]. Between the ages of 20 and 80 years the average reduction in muscle cross-sectional area amounts to 40 % [4]. The size and numbers of muscle fibers are under control of satellite cells, the muscle progenitor cells that lie inactivated between the basal lamina and the sarcolemma [5]. A decline in satellite cell number, together with type II muscle fiber atrophy has been reported to occur during aging [6, 7]. Morphologically, muscle fibers and satellite cells of elderly subjects show an accumulation of lipofuscin granules, a marker for oxidative damage [8, 9]. Despite its clinical importance, the pathophysiological mechanisms behind the development of sarcopenia are not yet well known.

A possible cause for sarcopenia is systemic low grade chronic inflammation. Increased systemic pro-inflammatory cytokine levels have been observed during aging and they have been associated recently with poor muscle strength independent of diseases, smoking or physical exercise [10, 11]. In patients suffering from rheumatoid arthritis (RA) the levels of inflammatory markers are high at middle age, despite anti-inflammatory treatment [12, 13]. In these patients, muscle strength is significantly lower compared with the general population [14, 15].

In this study we aimed to determine the impact of chronic systemic inflammation on age-related histological muscle characteristics, type II muscle fiber atrophy, the level of lipofuscin accumulation and satellite cell number per fiber. As a model for chronic systemic inflammation, we examined muscle biopsies from patients with RA who have a significantly higher pro-inflammatory profile when compared with patients with osteoarthritis (OA) [12, 16].

METHODS

Study Population

The study population included patients suffering from RA (n = 10) and OA as controls (n = 27) who underwent elective knee replacement surgery in the period 2008 to 2010. Patients with knee revision surgery, tumor surgery, acute trauma, osteonecrosis, myositis, or ankylosing spondylitis were excluded. RA patients were matched with OA patients on gender and age. Height and weight were assessed preoperatively, and inactivity (0 to 10) and pain scores (0 to 10) were assessed using the Dutch version of the AIMS questionnaire [17]. Preoperative blood samples were taken for measurement of C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR) and white blood cell (WBC) concentration. Anti-inflammatory medication use, including prednisone, methotrexate and TNF inhibitors during the 5 years before surgery and the presence of peripheral neuropathy and lumbar discopathy

were collected from medical charts. The study protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center.

Muscle Biopsies

A muscle biopsy was taken from the distal part of the vastus medialis muscle during elective knee replacement surgery. After embedding in Tissue-Tek (Sakura Finetek, the Netherlands) biopsies were frozen in liquid nitrogen and stored at −80 °C until further analysis. Muscle biopsies were cut in sections of 10 µm and 5 µm at −21 °C using a cryostat-microtome (Leica Instruments GmbH, Nussloch, Germany) and mounted on uncoated microscope slides (Starfrost, Braunschweig, Germany). Immediately after cutting, the sections were evaluated for cross-sectional alignment. Biopsies from RA and OA patients were stained simultaneously and blinded for patient characteristics.

Fiber Type Staining

To determine muscle fiber type, 10 µm sections were stored at −20 °C for a maximum of 1 night. The nonreversed method for ATPase staining as described by Round et al. was used [18]. Briefly, slides were incubated for 30 min in 10 mg ATP (ATP disodium salt, Sigma Chemicals Ltd) and dithiothreitol in buffered calcium chloride at pH 9.4 at 37 °C. Second, slides were washed in calcium chloride 1 % and incubated in cobalt chloride 2 % for 2 min. Afterward, slides were washed very thoroughly in distilled water and incubated in an ammonium sulphide 1 % solution for 30 s.

Lipofuscin Staining

Lipofuscin accumulation was determined using two 5-µm sections and the level of lipofuscin specific auto-fluorescence was determined [9]. To localize the cross-sectional muscle fibers, the fiber membrane was stained with PC128 (dilution 1:500, sheep anti-laminin, The Binding Site, Birmingham, U.K) as primary antibody and Cy5 (dilution 1:50) as secondary antibody. Stained sections were stored at 4 °C until images were captured.

Satellite Cell Staining

Satellite cells were stained as described earlier by Lindstrom and Thornell [19]. Two 5 µm sections were fixed in 2 % formaldehyde for 8 min and rinsed in 0.01 M phosphate buffered saline containing 0.05 % Tween 20 (VWR Prolabo, Fontenay-sous-bois, France) 3 times for 5 min each. Sections were blocked with IgG-free bovine serum antigen 4 % (Jackson Immuno Research, West Grove, Pennsylvania) for 90 min (first primary anti-body incubation) and for 60 min (second primary antibody incubation). First, sections were incubated with CD56 (dilution 1:3, mouse

anti-CD56; BD biosciences, San Jose, CA) overnight at 4 °C followed by incubation with FITC (dilution 1:50). Second, sections were incubated with Pax7 (dilution of 1:10, Mouse anti-PAX-7, Developmental Studies Hybridoma Bank, Iowa City, USA) and PC128 (dilution 1:500, Sheep anti-laminin, The Binding Site, Birmingham, UK) for 60 min at room temperature. PC128 were labeled with Cy5 (dilution 1:50) and Pax7 with Rhodamine Red (dilution 1:200). All secondary antibodies were purchased from Jackson Immuno Research, West Grove, Pennsylvania. Nuclei were stained with Hoechst 33258 (Molecular Probes, Leiden, The Netherlands) for 5 min at room temperature. Sections were washed with PBS 3 times for 5 min each, mounted with aqueous mounting medium (Dako, Carpinteria, USA) and covered with cover glasses. Slides were stored at 4 °C for a maximum of 3 days until images were captured.

Image Capture and Analysis

All images were captured and analyzed blindly for patient characteristics. Slides stained for muscle fiber type determination were scanned using a 3Dhistech automatic digital slide scanner (Panoramic Midi, 3Dhistech) with a 20× magnification (figure 1). For each patient a minimum of 200 (mean 339 for type I fibers and 596 for type II fibers) muscle fibers were randomly selected. The cross-sectional area of muscle fibers was measured using the program HistoQuant (3Dhistech). Fibers were counted using Image-J software, version 1.43. We quantified type II fiber atrophy by (1) the ratio of the mean area of single type II fibers to that of single type I fibers and (2) the number of type II fibers related to the total number of fibers. However, in 1 RA and 3 OA patients fiber type measurement was not possible due to poor staining quality or low number of muscle fibers.

Two hundred cross-sectional muscle fibers were captured at 40 × magnification to quantify lipofuscin accumulation using a conventional Leica DM 5500 B fluorescence microscope (Leica, Rijswijk, The Netherlands) together with a CoolSnap K4 cooled charge-coupled device camera (Roper Scientific, Evry Cedex, France). Contrast was enhanced, and the total gray value was measured using Image-J software version 1.43.

Satellite cells were visualized using the same fluorescence microscope and camera as was used for the lipofuscin measurements. At 40 × magnification images were captured and analyzed for at least 200 cross-sectional muscle fibers per patient. For this procedure the software program Colorproc was used to automatically decrease background staining. Satellite cell number was measured in 5 RA and 12 OA patients due to limited biomaterial. Cells were identified as satellite cells if they were stained by Pax7 or NCAM containing a nucleus stained by Hoechst and resided in a sublaminar position confirmed by the laminin

staining (figure 1). Satellite cells were identified and counted blinded to group by 2 independent observers. Disagreement was resolved by consensus. Satellite cell number was determined in relation to the number of muscle fibers within an image using the formula: number of counted satellite cells/number of counted muscle fibers × 100.

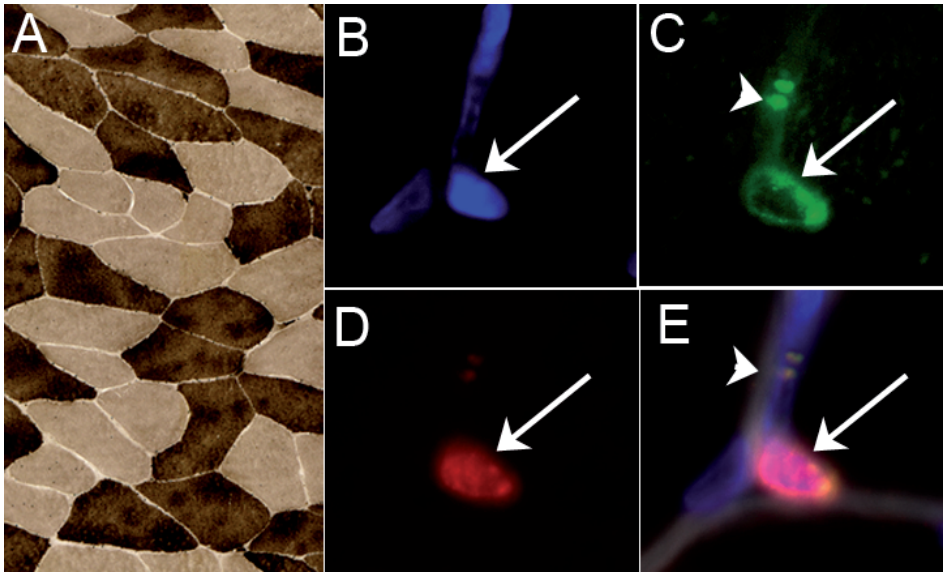

Figure 1: Images of muscle cross-sections stained for muscle fiber typing and satellite cell identification by multi-labeling
(A) Staining of type I (white) and type II (black) muscle fibers. (B) DAPI staining (blue). (C) N-CAM staining (green). (D) Pax7 staining (red). (E) Combination of DAPI, N-CAM, Pax7, and laminin (white). The satellite cell is indicated by an arrow. Lipofuscin granules are indicated by an arrowhead.

Statistical analysis

The independent-samples *t*-test was used to test for differences in age, height, weight, inactivity score, pain score, CRP, ESR, and WBC concentration between RA and OA patients. Differences in distribution of gender, the use of prednisone, methotrexate and TNF inhibitors and peripheral neuropathy and lumbar discopathy were tested by use of the Mann-Whitney test. Linear regression models were used to test for differences between RA and OA patients and for associations between muscle characteristics and CRP, ESR, and WBC concentration. All models were adjusted for age, gender, height and weight. Analyses were performed using SPSS software (version 16.0 SPSS Inc, Chicago, USA). All *p*-values below 0.05 were considered to be statistically significant.

RESULTS

The ratio of single type II and type I fiber areas, the percentage of type II fibers, the level of lipofuscin accumulation and the number of satellite cells per fiber were compared between patients with RA and OA. Patient characteristics are given in table I. The mean age of the RA group was 63.6 years (SD 9.1), and it was 66.0 years (SD 8.4) in the OA group. The gender distribution and pain scores were comparable between the groups. The inactivity score was not significantly higher in the RA group compared with the OA group. The mean duration of RA disease was 22.6 years (SD 13.0). All markers of inflammation as well as anti-inflammatory medication use were significantly higher in RA patients compared with OA patients.

Table I: Patient characteristics

	RA-group	OA-group	
Characteristic	n = 10	n = 27	*P* - value
Age (years)	63.6 (9.05)	66.0 (8.41)	ns
Sex (% female)	80	81	ns
Disease duration (years)	22.6 (13.0)	n/a	
Height (cm)	169.9 (11.7)	165.3 (6.7)	ns
Weight (kg)	85.0 (19.9)	86.4 (17.3)	ns
Clinical measurements *			
Inactivity score (0 – 10)	3.9 (2.9)	2.5 (2.0)	ns
Pain score (0 – 10)	5.4 (2.6)	6.6 (2.1)	ns
Markers of inflammation			
CRP (mg/l)	11.8 (10.8)	3.3 (4.9) †	0.038
ESR (mm)	23.8 (14.9)	10.2 (6.3) ‡	0.020
WBC (*10^9/L)	9.4 (2.8)	7.2 (1.5) §	0.042
Anti-inflammatory medication			
Prednisone (%)	3 (30)	1 (4)	0.024
Methotrexate (%)	8 (80)	0	< 0.001
TNF inhibitors (%)	3 (30)	0	0.003
Neurologic diseases			
Peripheral neuropathy (%)	0	2 (7)	ns
Lumbar discopathy (%)	1 (10)	1 (4)	ns

RA: rheumatoid arthritis, OA: osteoarthritis, CRP: C-reactive protein, ESR: Erythrocyte sedimentation rate, WBC: White blood cells, TNF: Tumor necrosis factor, ns: not significant. Values are given as mean (SD) if not otherwise stated. Dutch version of the AIMS questionnaire was used to assess the inactivity and pain scores. *available for 4 RA and 16 OA patients. †available for 18 OA patients. ‡available for 11 OA patients. §available for 22 OA patients.

Figure 2 shows the histological characteristics of the muscle tissue in patients with RA and OA. The mean ratio of type II and type I single fiber areas was 0.68 (SE 0.25) in the RA group versus 0.69 (SE 0.23) in the OA group. The mean percentage type II fibers was 66.8 % (SE 4.9) in the RA group and 62.7% (SE 1.6) in the OA group. The mean lipofuscin value was 0.21 (SE 0.03) in the RA group and 0.16 (SE 0.01) in the OA group. The number of satellite cells per fiber was 6.1 % (SE 2.9) in RA patients and 5.5 % (SE 1.1) in OA patients. After adjustment for gender, age, height and weight no significant differences in the ratio of type II and type I single fiber areas, the percentage of type II fibers, the level of lipofuscin accumulation and the number of satellite cells per fiber were found between RA and OA patients. Independent of RA and OA diagnosis, no significant association was found between markers of inflammation and histological muscle characteristics (table II).

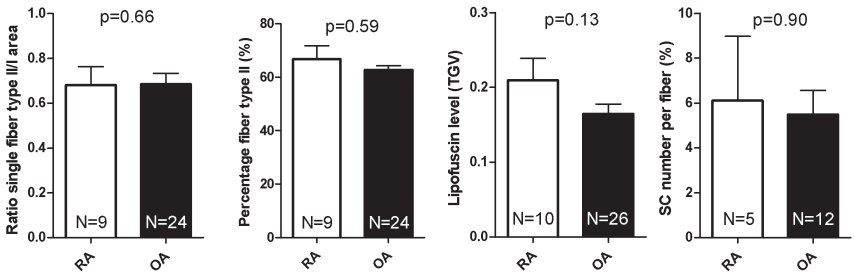

Figure 2: Ratio of type II and type I single fiber areas, percentage type II fibers, lipofuscin level and satellite cell number per fiber in muscle tissue from RA and OA patients
Results are given as mean and standard error. *p*-values are calculated using linear regression analysis adjusted for age, gender, height, and weight. RA: rheumatoid arthritis, OA: osteoarthritis, ratio of single type II/I area: ratio of the area of single type II fibers to that of single type I fibers, percentage type II fibers: number of type II fibers related to the total number of fibers, TGV: total gray value, SC: satellite cell.

DISCUSSION

Chronic systemic inflammation is suggested to play an important role in muscle wasting during aging [15, 20, 21]. To test for the impact of chronic systemic inflammation on characteristics associated with muscle aging, we compared muscle biopsies obtained from patients with an inflammatory disease (RA) and control patients (OA). No significant differences were found in type II muscle fiber atrophy, the level of lipofuscin accumulation or the number of satellite cells per fiber in patients with RA compared with OA patients.

Multiple factors, including age and inactivity, are known to cause type II muscle fiber atrophy [4, 22]. Both RA and OA are associated with a lower activity level and

Table II: Association between inflammatory markers and muscle characteristics

		Ratio single fiber type II/I area		Percentage fiber type II (%)		Lipofuscin (TGV) *		SC number per fiber (%) †	
Inflammatory marker	n	β (SE)	*p*	β (SE)	*p*	β (SE)	*p*	β (SE)	*p*
CRP (mg/l)	25	0.008 (0.006)	0.21	-0.35 (0.28)	0.23	-0.001 (0.002)	0.57	0.1 (0.3)	0.59
ESR (mm)	19	-0.002 (0.006)	0.72	0.25 (0.22)	0.28	0.001 (0.002)	0.63	-0.3 (0.3)	0.50
WBC (*10^9/L)	28	-0.004 (0.025)	0.86	1.05 (1.16)	0.37	-0.004 (0.009)	0.67	-0.7 (0.6)	0.27

Adjusted for age, gender, height, weight and rheumatoid arthritis and osteoarthritis diagnosis by linear regression. Ratio of single type II/I area: ratio of the area of a single type II fibers to that of single type I fibers. Percentage type II fibers: number of type II fibers related to the total number of fibers. TGV: total gray value, SC: satellite cell, n: number of patients, β: beta, SE: standard error, *p: p*-value, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, WBC: white blood cells.*n available for CRP: 28, ESR: 21, WBC: 32. † n available for CRP: 13, ESR: 9, WBC: 13.

type II muscle fiber atrophy [23-30]. Although the RA group had a slightly higher inactivity score compared with the OA group, neither the ratio of type II and type I single fiber areas nor the percentage of type II single fibers differed between groups. An earlier study compared muscle biopsies from RA and OA patients and found a significantly higher cross-sectional area of type II muscle fibers, but no significant difference in the ratio of type II and type I single fiber areas in 29 RA biopsies compared with 16 OA biopsies. The limitation of that study was that inactivity and pain levels were not measured [31]. We conclude that chronic systemic inflammation in RA has no additional contribution to type II muscle fiber type II.

Lipofuscin accumulation levels are a robust marker for past oxidative stress, which contributes to decline in muscle function in older adults [9, 32]. Reactive oxygen species have also been described to play a role in the pathophysiology of RA [33, 34]. A lower antioxidant enzyme activity and a higher level of oxidative damage products were observed in erythrocytes from RA patients compared with OA patients [34]. Compared with healthy controls, RA patients have a higher number of lipofuscin granules in the vastus lateralis muscle, when investigated by electron microscopy [35]. We found slightly higher levels of lipofuscin accumulation in patients with RA compared with patients with OA, but the difference was not statistically significant. It is possible that this difference would reach significance if more RA patients were included. Interestingly, physical inactivity due to OA has recently been associated with reduced mitochondrial function within skeletal muscle cells and with chronic inflammation [30]. A contribution of sedentary lifestyle to the accumulation of cellular damage cannot be excluded.

During chronological aging the number of satellite cells is known to decline [36]. Rodent studies have shown that high concentrations of inflammatory cytokines stimulate apoptosis of satellite cells *in vitro* [37]. A comparable study analyzing human satellite cell characteristics found that inflammatory cytokines stimulate the proliferation of satellite cells and inhibit their initiation of differentiation, but they do not induce apoptosis [38]. *In vivo*, it has been show that gene transfer of the pro-inflammatory factor TNF-alpha in mice causes a significant reduction in number and size of regenerating fibers following muscle injury [39]. Because RA patients are known to have higher concentrations of inflammatory markers compared with OA we hypothesized that this would lead to a reduced regenerative potential in RA, including a lower number of satellite cells. This is the first time that the number of satellite cells per fiber is quantified in patients with RA. Two studies examined RA muscle biopsies by electron microscopy. Using this method it is not possible to quantify satellite cells in relation to the number of fibers [40]. No satellite cells at all were found in intrafusal muscle fibers around muscle spindles in 100 RA patients [41]. Another study found a higher number of satellite cells in muscle biopsies from 12 RA patients compared with healthy controls [35]. We reported no difference in the number of satellite cells per fiber in RA and OA patients, indicating that a link between chronic systemic inflammation in RA and satellite cell number is unlikely.

Recently, it has been shown that higher CRP levels and a higher ESR in RA patients are associated with a lower lean body mass and the presence of sarcopenic obesity [42,43]. In this study, we found that type II fiber atrophy, lipofuscin accumulation, and satellite cell number per fiber were not significantly associated with ESR, CRP, or WBC concentration. Furthermore, it is known that several inflammatory cytokines are capable of accelerating muscle proteolysis [44]. However, the precise mechanism by which this would cause muscle wasting in RA or during the aging process has yet to be elucidated [45].

The strength of this study is the long disease duration and high levels of inflammation in RA patients compared with controls. Furthermore, both groups showed the same age and gender distribution. A limitation of this study is the relatively low number of patients with RA included in the satellite cell measurements. The effect of anti-inflammatory medication use like prednisone, methotrexate and TNF inhibitors could also have diluted the studied effect. In addition, OA patients are known to have higher levels of systemic inflammatory markers compared with healthy controls [46]. This low-grade inflammation in OA may have caused a substantial detrimental effect on skeletal muscle. Using OA as a control group for RA patients would therefore underestimate the impact of high-grade systemic inflammation on age-related muscle characteristics.

In conclusion, chronic systemic inflammation in RA is unlikely to be associated with type II muscle fiber atrophy, lipofuscin accumulation, or number of satellite cells per fiber. Further investigations should focus on chronic inflammation and satellite cell function such as activation and proliferation.

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AUTHOR CONTRIBUTIONS

Study concept and design: Westendorp, Nelissen, Maier. Patient inclusion and muscle biopsy handling: Beenakker, Duijnisveld, van der Linden, Visser, Maier. Staining protocol development: Beenakker, Duijnisveld, Butler-Brown, Maier. Analysis and interpretation of data: Beenakker, Duijnisveld, Maier. Drafting the manuscript: Beenakker, Duijnisveld, Maier. Critical revision of the manuscript for important intellectual content: Van der Linden, Visser, Westendorp, Butler-Brown, Maier. Statistical analysis: Beenakker, Maier. Study supervision: Westendorp, Maier. All authors approved the final version of the manuscript.

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Chapter 7

Regenerative potential of human muscle stem cells in chronic inflammation

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ABSTRACT

Background: Chronic inflammation is a profound systemic modification of the cellular microenvironment which could affect survival, repair and maintenance of muscle stem cells. The aim of this study was to define the role of chronic inflammation on the regenerative potential of satellite cells in human muscle.

Methods: As a model for chronic inflammation, 11 patients suffering from rheumatoid arthritis (RA) were included together with 16 patients with osteoarthritis (OA) as controls. The mean age of both groups was 64 years, with more females in the RA group compared to the OA group. During elective knee replacement surgery, a muscle biopsy was taken from the distal musculus vastus medialis. Cell populations from four RA and eight OA patients were used for extensive phenotyping because these cell populations showed no spontaneous differentiation and myogenic purity greater than 75 % after explantation.

Results: After mononuclear cell explantation, myogenic purity, viability, proliferation index, number of colonies, myogenic colonies, growth speed, maximum number of population doublings and fusion index were not different between RA and OA patients. Furthermore, the expression of proteins involved in replicative and stress-induced premature senescence and apoptosis, including p16, p21, p53, hTERT and cleaved caspase-3, was not different between RA and OA patients. Mean telomere length was shorter in the RA group compared to the OA group.

Conclusions: In the present study we found evidence that chronic inflammation in RA does not affect the *in vitro* regenerative potential of human satellite cells. Identification of mechanisms influencing muscle regeneration by modulation of its microenvironment may, therefore, be more appropriate.

INTRODUCTION

Muscle weakness is a common clinical feature following injury, in neuromuscular diseases and aging leading to disability and increased mortality [1-3]. Understanding cellular mechanisms that regulate loss and gain of muscle mass is, therefore, crucial for treating muscle wasting-associated disorders. The regenerative potential of skeletal muscle is determined by muscle stem cells, which are called satellite cells. These are quiescent mononucleated cells that are sequestered between the basal lamina and the plasma membrane of the myofibers [4]. In response to injury, they become activated, proliferate, differentiate and fuse to existing muscle fibers or fuse together to form new myofibers during regeneration of damaged skeletal muscle [5].

Potential explanations for the decline in muscle mass and strength are multiple factors, including stiffness, immobility, pain, metabolic, hormonal and nutritional status [6, 7]. These factors could influence muscle regeneration indicated by the number of satellite cells and their proliferative capacity, which in humans is limited by the mitotic clock [8, 9]. Heterochronic parabiosis has been shown to restore the regenerative potential of aged satellite cells which suggest that satellite cell activity can be modulated by the microenvironment and circulating factors [9]. There is growing evidence that chronic inflammation can produce a profound systemic modification of the cellular microenvironment which could affect survival, repair and maintenance of muscle cells [10]. Concentrations of pro-inflammatory cytokines have been shown to increase with advancing age and result in a higher catabolic rate and loss of muscle mass [11, 12]. The underlying factors influencing the regenerative potential of satellite cells have not yet been identified.

In the present study, we aimed to define the role of chronic inflammation on the regenerative potential of satellite cells in human muscle. As a model for *in vivo* chronic inflammation, we have used muscle biopsies obtained from patients suffering from rheumatoid arthritis (RA) compared to patients with osteoarthritis (OA), without signs of chronic inflammation. Patients with RA have been shown to have a steeper decline in muscle mass and strength compared to the general population, which might be due to the chronic inflammatory state of these patients [13, 14].

MATERIAL AND METHODS

Subjects

The total study population included 11 patients with RA and 16 patients with OA as controls. After obtaining written informed consent, a muscle biopsy (approximately 420 mg) was taken from the distal musculus vastus medialis during elective knee replacement surgery. Pre-operatively, blood samples were taken for analysis of C-reactive protein (CRP), using an immunoturbidimetric method, erythrocyte

sedimentation rate (ESR) using the Westergren method and the number of leucocytes using flow cytometry. The study was approved by the medical ethics committee of the Leiden University Medical Center.

Cell cultures

Myoblast explantation, isolation and cell cultures were performed as previously described [15-18]. Muscle biopsies from the musculus vastus medialis were dissected from connective tissue and fat, finely minced and then plated onto non-coated Petri dishes with growth medium consisting of Dulbecco's modified Eagle medium (DMEM, 61965, Invitrogen, Carlsbad, CA, USA), 16 % medium 199 (41150, Invitrogen), 20 % fetal calf serum (FCS, 10270, lot 41Q4096K, Invitrogen) and 50 ng/ml gentamicin (15750, Invitrogen) supplemented with 5 ng/ml hepatocyte growth factor (PHG0354, Invitrogen). Once mononucleated cells had migrated out from the explants, they were removed from the dish by trypsinization, using 0.05 % trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, 25300, Invitrogen). Mononucleated cells were filtered (40 µm) and plated as a mixed culture in growth medium. At the time of cell isolation, all cell populations were considered to be at one population doubling. All cultures were incubated at 37 °C in a humid air atmosphere containing 5 % CO_2. Cell populations were trypsinized when they reached 80% of confluence. Myogenic purity of the populations was determined by immunocytochemistry. To improve myogenic purity, cell populations were magnetically labelled by 15 minutes incubation with 20 µl CD56-micro beats per 10^6 cells (130-050-401, Multenyi Biotec, Paris, France). Cell populations were washed with buffer consisting of phosphate buffered saline (PBS, 20012, Invitrogen), 0.5 % FCS and 2 mM EDTA and CD56 cell selection was carried out using the MiniMACS (Multenyi Biotec). After determination of the myogenic purity, cell populations were frozen in 90 % FCS and 10% dimethyl sulphoxide hybri-max (D2650, Sigma-Aldrich, St. Louis, MO, USA) and preserved at -135 °C. Only cell populations with no spontaneous differentiation after trypsinization (less than 10 myotubes with more than 2 nuclei in a 100 mm petri dish) and myogenic purity greater than 75 % after CD56 cell selection were used for further analysis. To optimize the comparison of RA and OA patients, all experiments were performed in a highly standardized manner in three batches each containing at least one RA and one OA patient. At the early replicative phase (mean 10.3 (SD 1.7) population doublings (PDs)), myogenic purity, viability, proliferation index, colony formation and growth speed was measured. Protein analysis and telomere length analysis were performed at mean 14.4 (SD 3.1) PDs. Myogenic purity was again measured at the mid-term replicative phase (mean 24.3 (SD 4.2) PDs) and at the end of life span (maximum replicative capacity). The experiments were performed and analyzed blinded for patient diagnosis and age.

Myogenic purity

Myogenic purity of the cell cultures was determined by immunocytochemistry. Cells were rinsed in PBS, fixed with ethanol (100 %) and incubated for one hour with monoclonal mouse D33 (M0760, 1/50, Dako, Trappes, France) as primary antibody specific for desmin, which is only expressed in myogenic cells. Specific antibody binding was revealed in fluorescence by Alexa fluor 488 goat anti mouse antibody (1/750, A11029, Molecular Probes, Eugene, OR, USA) and nuclei were revealed by Hoechst (1/2500, 33258, Sigma-Aldrich) staining. Myogenic purity was calculated as the percentage of desmin positive cells divided by the total number of nuclei.

Growth characteristics

To measure the proliferation index, cells were cultured for 48 hours in culture medium supplemented with 10 µg/ml bromodeoxyuridine (BrdU, B5002, Sigma-Aldrich). Next, the cells were rinsed in PBS and fixed with ethanol (100 %). To render incorporated BrdU accessible to antibody, fixed cells were treated with 2 M HCl for 30 minutes at room temperature and were then neutralized by three 20-minute washes in 50 mM NaCl, 100 mM Tris HCl pH 7.5. Cells were incubated for one hour with a monoclonal antibody directed against BrdU (Bu20a, 1/40, M0744, Dako). Specific antibody binding was revealed in fluorescence by Alexa fluor 488 goat anti-mouse antibody and nuclei were revealed by Hoechst staining. The proliferation index was calculated as the percentage of BrdU positive cells divided by the total number of nuclei. The proliferation index was determined in three experimental triplicates counting at least 500 nuclei in each experiment.

Five hundred myoblasts were seeded on a 100 mm dish (353003, Becton Dickinson, Le Pont de Claix, France) and cultured for 14 days in growth medium to allow the formation of colonies. During this period the medium was not changed. Next, cells were rinsed in PBS, fixed with ethanol (100 %) and stained overnight with Giemsa's azur eosin methylene blue solution (109204, Merck, Darmstadt, Germany). Colonies were defined as a cluster of cells containing 16 cells or more. The total number of colonies formed was counted in experimental triplicates. The percentage of myogenic colonies was detected by immunocytochemistry using desmin (M0760, 1/50, Dako).

Growth speed was calculated by the number of PDs per day during the early replicative phase. The number of PDs at each passage was calculated as log(N/n)/log 2 where N is the number of cells counted at the time of passage and n is the number of cells initially plated. During long-term culturing, cells were continuously fed in a standardized manner by seeding 50,000 cells in a 100 mm dish (353003, Becton Dickinson) and serial passaged when they reached 80 % confluence until the end of their replicative lifespan. Cultures were considered to be senescent

when they failed to divide during three weeks of re-feeding or if the myogenic purity was decreased to less than 10 %.

Fusion index

To induce differentiation of the mononucleated cells into multinucleated myotubes, cells were densely seeded in triplicates on a 12-well dish (353043, Becton Dickinson, 17,000 cells/cm^2). Once confluent, growth medium was replaced by differentiation medium consisting of DMEM, 10 µg/ml insulin (I550-250 M, Sigma-Aldrich), 100 µg/ml transferrin (T4382-IG, Sigma-Aldrich) and 50 ng/ml gentamicin. After 120 hours, cells were rinsed in PBS and fixed with ethanol (100 %). To stain the myotubes, polyclonal mouse MF20 antibody (1/80, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was used as the primary antibody and Alexa fluor 488 goat anti mouse as the secondary antibody (A11029, 1/750, Molecular Probes). Nuclei were revealed by Hoechst staining. The fusion index was calculated as the percentage of nuclei incorporated into myotubes (> 2 nuclei) to the total number of nuclei. The fusion index was determined in three experimental triplicates counting at least 500 nuclei in each experiment.

Protein analysis

Cultures were washed in PBS, scraped in 1 ml ice-cold PBS, centrifuged at 800 rpm for one minute and cell pellets were stored at -80 °C for further analysis. Cell pellets were resuspended in 150 mL of RIPA buffer (150 mM NaCl, 5 mM EDTA, 50 mM HEPES, 0.5 % sodium deoxycholate, 1 % NP-40, 0.1 % SDS and complete mini-protease inhibitor) and protein concentration was determined with the Pierce BCA kit (Thermo Fisher Scientific, Rockford, IL, USA), using bovine serum albumin as a standard. Sample buffer (4 % SDS, 20 % glycerol, 0.2 M dithioethreitol, 125 mM Tris-HCl, pH 6.8) was added to aliquots of cell extracts and boiled for five minutes. Twenty µg of protein were loaded on 12 % SDS-polyacrylamide gels (SDS-PAGE). Following migration, proteins were transferred to nitrocellulose membranes. The proteins immobilized on the membranes were immediately blocked for one hour at room temperature with a solution containing 5 % non-fat dry milk in tris buffered saline containing 0.1 % tween 20 (PBT). The blocked membranes were then incubated with a rabbit polyclonal anti-p16 antibody (1/1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-beta-tubulin antibody (1/1000, clone TUB 2, T4026, Sigma-Aldrich) as the primary antibody and anti-rabbit or anti-mouse peroxidase conjugated antibody (1/40.000, GE Healthcare, Orsay, France) as the secondary antibody. Anti-p16 and anti-tubulin antibodies were visualized using the ECL plus Western Blotting Detection System (GE Healthcare). For p21, p53, cleaved caspase-3 and hTERT detection, PDVF membranes were stained for one minute with 0.1 % Ponceau (Sigma-Aldrich). Mouse anti-p21 antibody (CP74,

1/250, kindly provided by A.G. Jochemsen of the Department of Molecular Cell Biology, LUMC), mouse anti-p53 antibody (1/500, Santa Cruz Biotechnology), rabbit anti-human-cleaved caspase-3 (H269518, 1/250, R&D Systems, Abingdon, UK), rabbit anti-hTERT (1531-1, 1/500, Epitomics, Burlingame, CA, USA) and monoclonal mouse anti-muscle actin (1/1000, Santa Cruz Biotechnology) were used as primary antibodies and fluorochrome conjugated goat anti-mouse or goat anti-rabbit as secondary antibodies (1/2000, Licor Biosciences, Lincoln, NE, USA). Quantification of protein expression was performed using ImageJ (http://rsb.info.nih.gov/ij/) with data obtained from two experiments. Tubulin or Actin signals were used for normalization.

Telomere length

Telomere length was measured by flow cytometry using fluorescence *in situ* hybridization with fluorescein-conjugated peptide nucleic acid (PNA) as the probe (K5327, Dako) according to the manufacturer's protocol. In short, cultured cells were trypsinized, mixed with the reference cell line (line 1301; Banca Biologica e Cell Factory, Genoa, Italy), and hybridized with and without Cy3-labeled peptide nucleic acid probe. After labelling the cells with propidium iodide (PI) for DNA content, acquisition was performed using a FacsAria flow cytometer (Becton Dickinson) equipped with Diva software. The probe signal was measured in the FL-1 channel and the propidium iodide signal in the FL-3 channel. Experimental duplicates were performed and results were analyzed according to the manufacturer's protocol.

Oxidative stress

Twenty-four hours after seeding, cell cultures were exposed to a single acute oxidative stress of 250 µM H_2O_2 (H-1009, Sigma-Aldrich) diluted in DMEM with 20 % medium 199 and 50 ng/ml gentamicin for 60 minutes at 37 °C. Next, cell cultures were rinsed with PBS and cultured in growth medium. Control and stressed cell cultures of three RA and four OA patients followed the same treatment protocol in triplicates with and without the addition of H_2O_2 respectively. During the early replicative phase (mean 10.3 (SD 1.7) PDs), myogenic purity, viability, proliferation index, colony formation and growth speed were measured. The percentage of viable cells was determined 24 hours after oxidative stress by trypan blue (T8154, Sigma-Aldrich) exclusion.

Statistical analysis

Data are presented as mean and standard deviation. Experimental triplicates or duplicates were averaged for statistical analysis. A linear regression model was performed with adjustment for age and gender, myogenic purity, number of PDs and batch. The *p* - value tested the null hypothesis that the diagnosis (RA) does

not influence the outcome parameter. All *p*-values below 0.05 were considered statistically significant.

RESULTS

To investigate the regenerative potential of satellite cells in chronic inflammation, 11 RA patients were included in this study together with 16 OA patients as controls who were all scheduled for elective knee replacement surgery (table I). The mean age of both groups was 64 years, with more females in the RA group (91 %) compared to the OA group (56 %). The mean disease duration in the RA group was 16 years (SD 13). Pre-operative blood samples showed a higher level of inflammatory markers, including a higher CRP, ESR and leucocyte level compared to the OA group. During elective knee replacement surgery, a muscle biopsy (approximately 420 mg) was taken from the distal musculus vastus medialis. After mononuclear cell explantation, the mean myogenic purity of the RA group was 34 % (SD 20) with 18 % spontaneous differentiation in the cell cultures compared to 46 % (SD 26) mean myogenic purity in the OA group with 19 % of the samples showing spontaneous differentiation. Cell strains without spontaneous differentiation of satellite cells and myogenic purity greater than

Table I: Characteristics of patients and satellite cell isolation

	Total study population		Selected study population	
	RA n = 11	OA n = 16	RA n = 4	OA n = 8
Age (years)	64 (12)	64 (9)	65 (9)	64 (9)
Gender (% female)	91	56	75	50
Disease duration (years)	16 (13)	-	15 (13)	-
CRP (mg/L)	12 (11)	2 (3)	10 (7)	2 (3)
ESR (mm)	28 (13)	11 (6)	28 (15)	11 (7)
Leucocytes (*10^9/L)	10.5 (2.5)	7.0 (1.6)	9.7 (1.4)	7.2 (1.6)
Myogenic purity after explantation (% desmin positive cells)	34 (20)	46 (26)	46 (13)	58 (26)
Spontaneous differentiation * (%)	18	19	0	0
Sufficient biopsy quality # (%)	36	50	100	100

CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, n: number of subjects, OA: osteoarthritis, RA: rheumatoid arthritis. Values are given as mean (SD) if not otherwise stated. CRP and leukocyte data were available for 14 and 10 patients in the OA and RA group respectively. ESR data were available for 7 patients in OA group and 10 patients in the RA group. *Spontaneous differentiation was defined as 10 or more myotubes with more than 2 nuclei in a 100 mm culture dish. #Sufficient biopsy quality was defined as no spontaneous differentiation and myogenic purity higher than 75 % after CD56 cell selection.

75 % after CD56 cell selection were considered to be sufficiently pure for further experiments. The resulting experimental group included four RA and eight OA patients. As shown in table I, this selected study population had the same distribution in age, gender, disease duration and inflammatory markers as the total study population.

Satellite cell characteristics

To investigate the influence of chronic inflammation on human satellite cell regenerative potential, multiple satellite cell characteristics were measured (table II, figure 1). During the early replicative phase (mean 10.3 (SD 1.7) PDs) myogenic purity, viability, proliferation index, mean number of colonies,

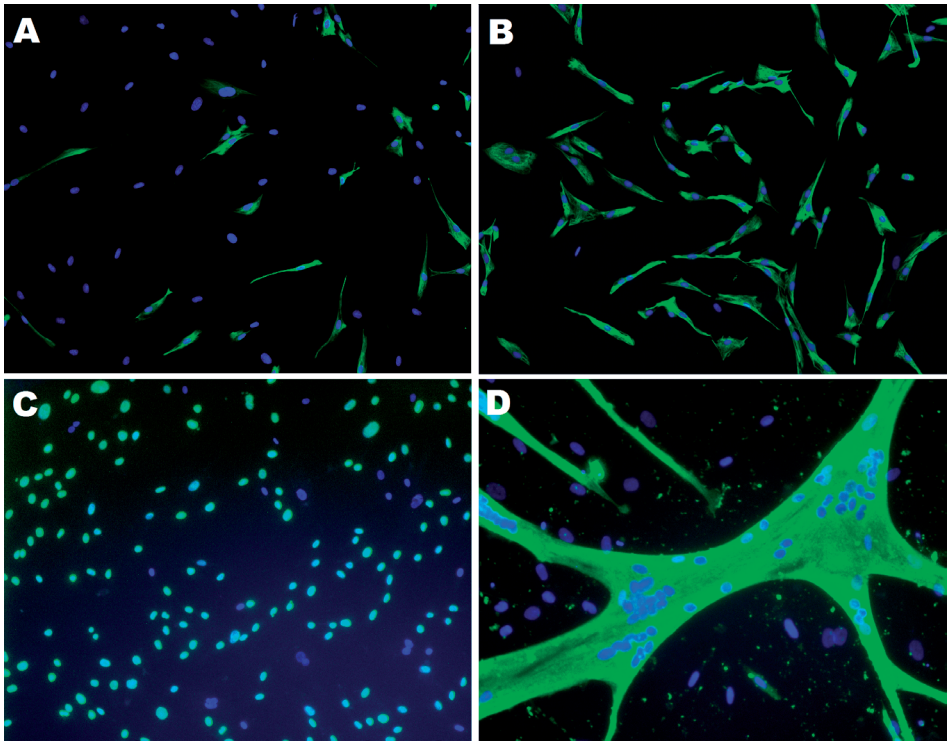

Figure 1: *In vitro* characterization of human satellite cells
Representative images of *in vitro* characterization of cultured myoblasts using immunofluorescence with an antibody against desmin (A and B), BrdU (C) and MF20 (D). Specific antibody labeling was revealed using Alexa fluor 488 green fluorochrome. Nuclei were visualized with Hoechst (blue). Myogenic purity was determined after explantation (A) and increased after CD56 selection (B). To determine the proliferation index, cells were cultured for 48 hours in culture medium supplemented with 10 µg/ml BrdU (C). To determine the fusion index, cells were cultured in differentiation medium for 120 hours (D). Original magnification 100 x (A, B and C) and 100 x (D).

Table II: Characteristics of satellite cell strains obtained from rheumatoid arthritis and osteoarthritis patients

Characteristic	RA n = 4	OA n = 8	β	95 % CI	*p* - value
Myogenicity (% desmin positive cells)					
After explantation	46 (13)	58 (26)	-0.35	-51 to 19	0.32
After CD56 selection	87 (5)	95 (3)	-0.72	-13 to -2	0.02
After thawing at early replicative phase *	60 (20)	47 (32)	-0.57	-209 to 142	0.62
At mid-term replicative phase #	56 (37)	37 (33)	-0.02	-49 to 46	0.95
At maximum replicative capacity	47 (43)	38 (30)	-0.07	-86 to 77	0.90
After oxidative stress †	21 (12)	16 (10)	0.21	-341 to 349	0.91
Viability (% alive cells)					
At early replicative phase *	91 (4)	94 (2)	-0.39	-8 to 4	0.33
After oxidative stress †	33 (24)	34 (24)	0.48	-114 to 153	0.31
Growth characteristics *					
Proliferation index ‡ (% BrdU positive cells)	86 (2)	85 (6)	-0.05	-10 to 9	0.89
Colony formation (number)	78 (44)	85 (34)	-0.12	-83 to 66	0.78
Myogenic colonies (%)	30 (16)	27 (31)	-0.40	-41 to 7	0.14
Growth speed (PD/day)	0.39 (0.10)	0.32 (0.08)	0.31	-0.07 to 0.18	0.29
Maximum replicative capacity (PD)	33.4 (9.7)	27.4 (5.5)	0.28	-5.0 to 13.9	0.28
Proliferation index after oxidative stress †‡ (% BrdU positive cells)	15 (15)	34 (24)	-0.29	-385 to 359	0.73
Fusion index at early replicative phase *§ (% of fused myonuclei)	27 (12)	25 (13)	-0.01	-26 to 26	0.99
Normalized protein expression *					
p16	103 (15)	140 (63)	-0.62	-85 to 7	0.08
p21	27 (4)	27 (5)	-0.28	-13 to 8	0.60
p53	67 (12)	62 (8)	-0.18	-18 to 11	0.58
hTERT	32 (6)	39 (14)	-0.26	-23 to 11	0.39
Relative telomere length * (% of ref. cell line 1301)	22 (5)	23 (10)	-0.46	-16 to -0.4	0.04

n: number of subjects, OA: osteoarthritis, RA: rheumatoid arthritis. Beta (β). 95 % confidence interval (CI) and *p*-value are shown for linear regression model adjusted for age and gender, myogenic purity, number of PDs, and batch. Values are given as mean (SD) if not otherwise stated. *Early replicative phase: mean 10.3 (SD 1.7) PDs for myogenic purity, viability, growth characteristics and fusion index; mean 14.4 (SD 3.1) PDs for normalized protein expression and relative telomere length. #Mid-term replicative phase: mean 24.3 (SD 4.2) PDs. †Myogenic purity, viability and proliferation index was measured at early replicative phase, 24 hours after oxidative stress (one hour of 250 μM H_2O_2 at 37 °C) in four OA and in three RA patients. ‡Proliferation index was determined as the percentage of bromodeoxyuridine (BrdU) positive cells after 48 hours of culture with BrdU. §Fusion index was determined as the percentage of fused nuclei (> 2 nuclei per myofiber) in MF20 positive cells.

myogenic colonies, growth speed and fusion index were not different between RA and OA patients as was the myogenic purity at mid-term and at replicative senescence. As depicted in figure 2, the growth curves showed no difference between the RA group with a mean maximum number of 33.4 (SD 9.7) PDs and the OA group with mean maximum of 27.4 (SD 5.5) PDs. After adjustment for age, gender, myogenic purity, number of PDs and batch, none of these parameters were statistically significantly different between RA and OA patients (data not shown).

Senescence-associated proteins apoptosis and telomere length

To determine whether senescence is induced in satellite cells, the expression of hTERT, p53, p21 and p16 proteins was determined at the early replicative phase using a Western blot analysis. Averages of normalized protein expression did not significantly differ between RA and OA patients (table II, figure 3). In addition, none of the samples were positive for cleaved caspase-3 expression. Furthermore, the mean relative telomere length was not different between RA and OA patients (Table II, Figure 4). After adjustment for age, gender, myogenic purity and batch, the mean relative telomere length was lower in the RA group compared to the OA group (β = -0.46, 95 % CI -16 to -0.4, p = 0.04). The other measurements were not different. The maximum replicative capacity of the

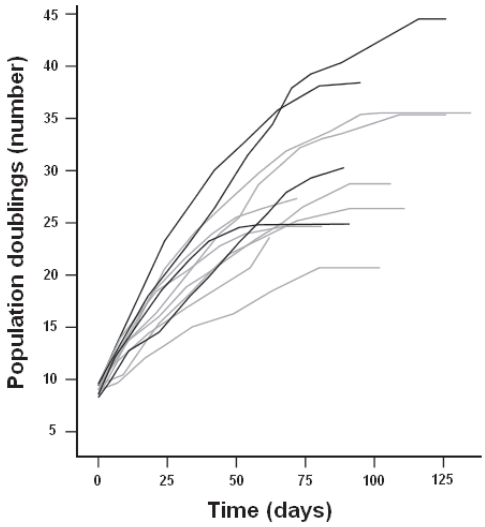

Figure 2: Growth curve of human satellite cells
Growth curve from thawing (day 0) until maximum replicative capacity of human satellite cell strains from patients suffering from rheumatoid arthritis (n = 4, black lines) or osteoarthritis (n = 8, gray lines).

satellite cell strains was not significantly associated with telomere length (β = 0.60, 95 % CI -1.3 to 2.3, *p* = 0.48).

Oxidative stress

To determine whether satellite cells from RA patients were more susceptible to oxidative stress, we administered a single dose of 250 μM H_2O_2 for 60 minutes at 37 °C to the cell cultures. Twenty-four hours after oxidative stress, the myogenic purity, viability and proliferation index were decreased in both the RA and OA group. None of the cell strains were able to reach confluence after three weeks or to form colonies after oxidative stress. The satellite cells from the RA group were as susceptible to oxidative stress as the OA group as shown in table II, also after adjustment for age, gender, batch and myogenic purity (data not shown).

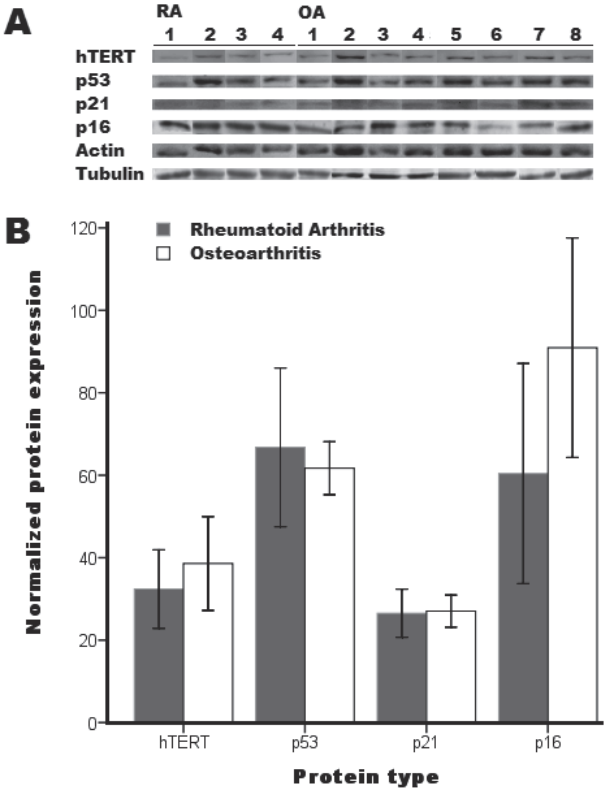

Figure 3: Protein expression of human satellite cells
A: Representative image of hTERT, p53, p21 and p16 expression in human satellite cells from patients suffering from rheumatoid arthritis (n = 4) or osteoarthritis (n = 8). Actin and tubulin expression was used for normalization. B: Normalized average protein expression and 95 % confidence interval of hTERT, p53, p21 and p16.

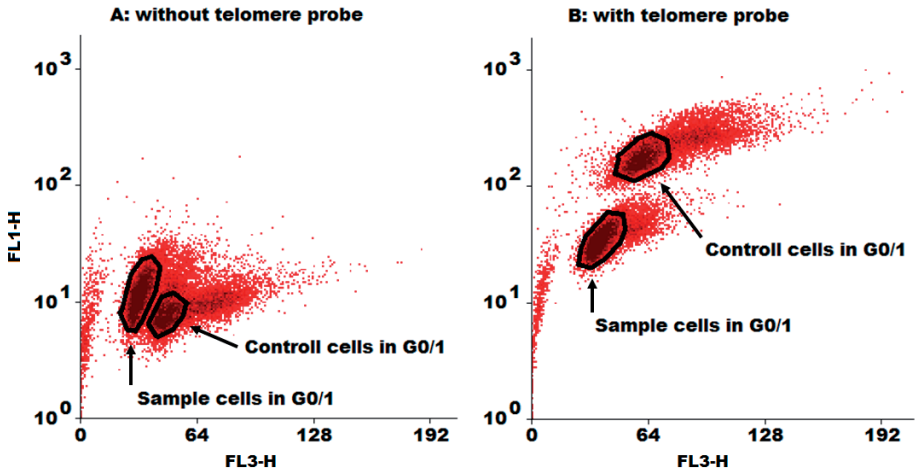

Figure 4: Telomere length of human satellite cells
Telomere length was measured by flow cytometry using fluorescence *in situ* hybridization without (A) or with the fluorescein-conjugated PNA probe (B). Propidium iodide (PI) was used for labeling DNA content. The telomere probe signal was measured in the FL-1 channel and the PI signal in the FL-3 channel. G0/1 phase sample and control cells were gated according to their PI specific signal on a dot blot (FL1-H vs. FL3-H). Relative telomere length (RTL) was calculated by: RTL = (mean FL1 sample cells with probe – mean FL1 sample cells without probe) / (mean FL1 control cells with probe – mean FL1 control cells without probe) * 2 * 100 %. Representative images are shown of the one of the RA patients. For each sample analyzed 20,000 counts were acquired.

DISCUSSION

The regenerative potential of human muscle stem cells is thought to be influenced by their microenvironment, including cytokines. To determine the potential deleterious effect of inflammation on the regenerative capacity of skeletal muscle, we compared muscle biopsies obtained from patients with chronic inflammation (RA) to control patients (OA). We found no differences in myogenic purity, viability, growth speed, differentiation and maximum proliferative capacity. These results were confirmed by no difference in major pathways involved in muscle regeneration, including the expression of proteins involved in replicative and stress induced premature senescence and apoptosis. Telomere length was shorter in RA patients compared to controls.

Mechanisms involved in the regenerative potential of muscle include the ability to restore the quiescent satellite cell pool which has been shown to decrease during aging by a decrease in the number of satellite cells present in muscle biopsies [9, 14]. Recently, we have shown that chronic inflammation does not influence the number of satellite cells in human muscle [19]. In the present study, we provide new evidence that the *in vitro* regenerative potential of human satellite cells seems not to be

affected by chronic inflammation. Mechanisms affecting the regenerative potential of muscle include the number of satellite cells which are able to proliferate, their growth speed and their maximum proliferative capacity which have been shown to be affected by age [8]. In the present study we demonstrate *in vitro*, that chronic inflammation *in vivo* does neither affect any of the growth characteristics nor the differentiation potential of human satellite cells. Regenerative potential of human satellite cells is limited by the progressive erosion of their telomeres after each cell division leading to critically short telomere length and the activation of replicative senescence through a p53 and p21 dependent pathway [20]. The erosion of telomeres can be prevented by the catalytic subunit of the telomerase (hTERT) leading to extension of replicative life [21]. The mean relative telomere length in RA patients was significantly lower after correction for age, gender, myogenic purity, batch and number of population doublings, which could suggest an increased turnover of these cells. However, this difference was minimal and the expression of p53, p21 and hTERT proteins were not different between RA and OA patients. Therefore, we conclude that chronic inflammation does not significantly increase nuclear turnover which is in agreement with our findings regarding the growth characteristics of satellite cells cultures. Stress induced premature senescence has been shown to be induced by p16 in aging and myotonic dystrophy [17, 22]. Here we demonstrate that p16 is not increased in chronic inflammation. Apoptosis and programmed cell death was not detected during the amplification of either chronic inflammation (RA) or control cells (OA) using anti-cleaved caspase 3 [23].

An unexpected finding was the low mean myogenic purity after explantation in both groups, as myogenic purity was shown to be high in both young and old age, which could have been due to the different biopsy material and procedures used in the different studies [8]. To optimize myogenic purity, we performed a CD56 magnetic selection procedure and only cell populations with a mean myogenic purity higher than 75 % were included in our study. Some cell cultures showed spontaneous differentiation and were, therefore, excluded. Because of low myogenic purity and increased spontaneous differentiation, we were able to obtain fewer biopsies in the RA group than in the OA group for *in vitro* experiments. Although the difference in muscle biopsy quality was not significant, this could indicate that chronic inflammation does influence satellite cell behavior and ultimately the regenerative potential of the muscle. Non-myogenic stem cells have, however, been shown to contribute to muscle regeneration [24]. After thawing, we also observed a decrease in mean myogenic purity in both groups. Freezing could have led to stress by cold shock, ice formation, activation of apoptosis mechanisms and alteration of certain signalling pathways. Fetal and newborn muscle cells have been shown to conserve their high myogenic purity after long-term cryopreservation; however,

adult cells are more difficult to explant and have a lower myogenic purity [25]. In the present study, we did not observe a correlation between myoblast telomere length and replicative capacity. Furthermore, we did not observe a correlation between telomere length and other markers of replicative capacity like the expression of p21, p53 or hTERT. A potential explanation involves the induction of pathways involved in stress induced senescence, being telomere length independent, which cannot be excluded during *in vitro* replicative senescence [26]. Serial culturing represents a major difference from the *in vivo* environment, including changes in their physical environment, the cell to cell interaction and different nutrition supply, which could induce oxidative stress, DNA damage and, finally, stress induced premature senescence [27, 28].

As a model for chronic inflammation, the study included patients suffering from RA who indeed showed a higher level of inflammation compared to OA patients as indicated by CRP, ESR and number of leucocytes. Furthermore, the two groups showed the same age distribution. Although OA is associated with local inflammation [29], we did not use a healthy control group because such a control group is physically more active, which would then possibly confound the results [30]. Furthermore, healthy patients without an underlying chronic condition are not available for the same muscle biopsy area, technique and biopsy size. The strength of this study is the rigorous standardization in terms of patient selection, muscle biopsy location, cell culture experiments in batches with both RA and OA patients and the extensive phenotyping of the satellite cell populations. The limitations of this study are that the influence of chronic inflammation on the *in vitro* regenerative potential of human satellite cells may not be found due to the selection of muscle biopsies with good quality. Secondly, the *in vitro* experiments might not reflect the *in vivo* situation and finally the small number of included patients made it impossible to differentiate within and between the groups on the presence of biological treatment (for example, anti TNF). In addition, it should be noted that there was a certain selection since the myogenic purity was lower in the RA group and many cells were lost to spontaneous differentiation which could be due to an increased expression of p16. However, including more patients was not possible due to the extensive phenotyping of each satellite cell strain. Furthermore, the RA group included more females (75 %) compared to the OA group (50 %). Myogenic purity and gender were adjusted for in the linear regression model and had no effect on satellite cell regenerative potential *in vitro*.

Muscle weakness is an increasing clinical problem and occurs upon injury, neuromuscular diseases and aging, leading to disability and increased mortality [1-3, 31, 32]. Stem cell therapy is one possible strategy to regenerate muscle. Although many studies with different types of stem cells have been conducted, the efficacy of

cell therapy is still limited by homing and a reduced ability to resist the environment of the damaged muscle [33]. Conboy *et al.* showed that heterochronic parabiosis could restore the regenerative capacity of aged satellite cells [34]. The circulating factors presented by a shared circulatory system could be the possible explanation for the observed rejuvenation. Therefore, the microenvironment of muscle in which pro-inflammatory cytokines have been shown to result in a higher catabolic rate and loss of muscle mass might be crucial for the regenerative potential [11, 12]. One of the hallmarks of inflammation is the release of interleukins and tumour necrosis factor-α (TNFα) attracting macrophages which are critical in the repair of skeletal muscle by the release of cytokines and growth factors and by phagocytosis. However, in their attempt to enhance tissue repair they may also destroy surrounding healthy muscle fibers [35]. Furthermore, TNF-α induces activation of transcription factor NF-κB, which results in activation of the ubiquitin-proteasome pathway and breakdown of MyoD and myogenin, which are essential regulators for satellite cell differentiation [36] as well as contractile proteins essential in maintaining muscle mass. Inflammatory factors could be the cause of lower muscle strength of patients with RA compared to OA [14].

In vitro, our results demonstrate no difference in potential regenerative capacity between satellite cells from patients with chronic inflammation and control patients. This could be due to the fact that the satellite cells of both patient groups were put in culture and, therefore, both experienced an optimized microenvironment for proliferation and differentiation. Consequently, our results show that there is no difference in satellite cell behavior *in vitro*, indicating that circulating inflammatory factors *in vivo* could be the cause of the decrease in muscle strength. Further evidence of the *in vivo* influence of inflammatory cytokines has been shown by anti-inflammatory treatment, which has been shown to improve muscle regeneration after injection with mesoangioblasts [37].

In conclusion, in the present study we found evidence that chronic inflammation in RA does not affect the *in vitro* regenerative potential of human satellite cells. This result underscores the *in vivo* influence of inflammatory factors on muscle regeneration. Identification of mechanisms influencing muscle regeneration by modulation of its microenvironment may improve strategies to regenerate human muscle, in particular in conditions such as muscle injury, neuromuscular disorders and aging.

ABBREVIATIONS

BrdU, bromodeoxyuridine; CI, confidence interval; CRP, C-reactive protein; DMEM, Dulbecco's modified Eagle medium; ESR, Erythrocyte sedimentation rate; FCS, fetal calf serum; N, number of subjects; OA, osteoarthritis; PBS, phosphate buffered

saline; PDs, population doublings; PI, propidium iodide; PNA, peptide nucleic acid; RA, rheumatoid arthritis; SD, standard deviation; TNFα, tumour necrosis factor-α

COMPETING INTERESTS

The authors have declared no competing interests.

AUTHORS' CONTRIBUTIONS

BJD, GS B-B, RGJW, RGHHN and ABM developed the study concept and design. BJD, ABM, KGMB, HJLvdH, CPJV and RGHHN were responsible for patient inclusion and muscle biopsy handling. KM, SC and AB were responsible for cell culture MACS, immunocytochemistry before and after purification, tests for mycoplasm, and amplification, freezing and storage of cell samples. BJD, AB and DMP acquired the data. BJD, AB, GS B-B, VM, DMP, VR, RGHHN and ABM analyzed and interpreted the data. BJD and ABM drafted the manuscript. AB, GS B-B, VM, DMP, VR, KGMB, HJLvdH, CPJV, RGJW, RGHHN and ABM provided critical revision of the manuscript for important intellectual content. BJD and ABM carried out statistical analysis. GS B-B, RGJW, RGHHN and ABM supervised the study. All authors approved the final version of the manuscript.

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Chapter 8

Local injection of autologous bone marrow cells to regenerate muscle in patients with traumatic brachial plexus injury: a pilot study

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ABSTRACT

Introduction: Traumatic brachial plexus injury causes severe functional impairment of the arm. Elbow flexion is often affected. Nerve surgery or tendon transfers provide the only means to obtain improved elbow flexion. Unfortunately, the functionality of the arm often remains insufficient. Stem cell therapy could potentially improve muscle strength and avoid muscle-tendon transfer. This pilot study assesses the safety and regenerative potential of autologous bone marrow-derived mononuclear cell injection in partially denervated biceps.

Methods: Nine brachial plexus patients with insufficient elbow flexion (i.e., partial denervation) received intramuscular escalating doses of autologous bone marrow-derived mononuclear cells, combined with tendon transfers. Effect parameters included biceps biopsies, motor unit analysis on needle electromyography and computerised muscle tomography, before and after cell therapy.

Results: No adverse effects in vital signs, bone marrow aspiration sites, injection sites, or surgical wound were seen. After cell therapy there was a 52 % decrease in muscle fibrosis ($p = 0.01$), an 80 % increase in myofibre diameter ($p = 0.007$), a 50 % increase in satellite cells ($p = 0.045$) and an 83 % increase in capillary-to-myofibre ratio ($p < 0.001$) was shown. CT analysis demonstrated a 48 % decrease in mean muscle density ($p = 0.009$). Motor unit analysis showed a mean increase of 36 % in motor unit amplitude ($p = 0.045$), 22 % increase in duration ($p = 0.005$) and 29 % increase in number of phases ($p = 0.002$).

Conclusions: Mononuclear cell injection in partly denervated muscle of brachial plexus patients is safe. The results suggest enhanced muscle reinnervation and regeneration.

INTRODUCTION

Brachial plexus injury (BPI) is often a result of a high-energy road traffic accident in young adults [1, 2]. The upper brachial plexus (C5-C6) is commonly affected, resulting in paresis of shoulder function and elbow flexion [1-4]. Nerve surgery is aimed at restoring innervation of the biceps muscle, but often a deficit in the functionality of the arm remains. In that case, secondary surgery can improve arm function by transfer of the flexor-pronator group of the forearm (Steindler procedure) for active elbow flexion [5-10]. However, neither nerve surgery nor tendon transfers come close to restoring normal elbow flexion [6].

Any procedure that improves elbow flexion without sacrificing the function of transferred donor muscles will have advantages for the patient. One approach is to improve the regenerative capacity of the partially denervated muscles. Denervation causes loss of contractile force and muscle atrophy. If innervation is restored quickly, good restorative ability exists. Conversely, prolonged interruption of innnervation results in irreversible muscle atrophy, interstitial fibrosis and muscle fattening [11-14]. Since regeneration of denervated muscles not only depends on nerve supply, but also on its regenerative capability, the muscle can be a target organ for cell therapy.

Muscle satellite cells are responsible for repair and maintenance of skeletal muscles; they are the main source of new myofibres. Myogenic precursor cells proliferate, fuse and form new myofibres in response to muscle damage [15]. Exhaustion of the pool of available satellite cells may contribute to poor functional recovery of long-term denervated muscle [16, 17]. A progressive and rapid decrease in the capillary-to-muscle fibre ratio occurs, further limiting regeneration [18]. Transplantation of primary satellite cells has been shown to improve the properties of reinnervated skeletal muscles in rabbits [19]. However, poor cellular survival and limited cell dissemination hampers successful satellite cell transplantation. Furthermore, only few cells fuse with host fibres. This suggests that a subpopulation of myogenic cells (i.e. stem cells) may be optimally suited for transplantation [20, 21]. Bone marrow (BM)-derived cells migrate to the site of muscle injury and contribute to the satellite cell pool [22-24]. The injection of autologous BM-derived mononuclear cells (MNCs) has been applied in clinical studies focusing on the muscles of the heart and leg [25-28].

The primary objective was to assess the safety of autologous BM-derived MNC injection in partially denervated muscles of BPI patients. The secondary objective was to obtain a first estimate on regenerative potential of injected BM-derived MNCs in partially denervated muscles.

METHODS

Study design

This was a prospective study on autologous BM-derived MNC therapy for partially denervated biceps muscles of BPI patients, with focus on safety and the effect on muscle. Three escalating doses were evaluated in three groups, each comprising three patients. The MNC dose was equivalent to 50 % (group A), 100 % (group B), and 200 % (group C) of the cell dose in a former study at our institution [25]. The protocol was approved by the medical ethics committee of Leiden University Medical Center. The Declaration of Helsinki protocols were followed and patients gave written, informed consent. The clinical trial was registered under ClinicalTrials.gov identifier: NCT00755586.

Patients

Nine adult traumatic BPI patients with partial denervation of the biceps muscle (Medical Research Council (MRC) grades between 1 and 3) were included [29]. All patients were at an end-stage of functional results (i.e. ≥ 2 years after trauma or nerve surgery). Patients with a complete paralysis of the biceps muscle were excluded as they were assumed to have no regenerative capacity (MRC grade 0). This was corroborated by the absence of motor unit potentials (MUPs) during needle electromyography (EMG). Other exclusion criteria were a history of central or other peripheral neurological disorders, humeral fractures, and contraindication for BM harvesting (such as bleeding diathesis or an international normalised ratio (INR) > 2).

BM aspiration and MNC separation

BM was aspirated from one location at the posterior iliac crest. The procedure was performed under local (group A) or general anaesthesia (groups B and C). Due to ethical considerations associated with the use of general anaesthesia, in groups B and C the MNC injection was combined with a Steindler procedure, which was considered the standard surgical procedure in patients with an elbow flexion deficiency [5]. BM was separated as previously described [25, 28]. The isolated MNCs were concentrated in a volume of 10 ml. The isolation and concentration procedure was performed in a certified clean-room facility according to good manufacturing practice (GMP) [25, 28]. Further details of the methods are provided as supplementary material.

MNC injection

BM-derived MNCs were injected at 20 sites at the maximum palpable thickness of the biceps and at a standard injection depth of 0.5 cm. A total volume of 10 mL was injected. Further details of the methods are provided as supplementary material.

Safety

In order to assure safety, the absence of adverse events and muscle fibrosis was documented before proceeding to the next dose level. Vital signs were checked and the BM aspiration, MNC injection and surgical wound site were examined during a 24-hour hospital admission and at three and six months after cell therapy. The safety of MNC injection at muscle level was assessed by CT analysis and histology.

Evaluation of patients

All patients were evaluated pre-operatively and at three and six months post-operatively using clinical scores, CT analysis and EMG. Histology of the biceps muscle was undertaken pre-operatively and at three months post-operatively. Before analysis, all images were blinded for date and patients characteristics.

Clinical functionality

Active and passive range of movement (ROM) of the injured elbow was measured using a handheld goniometer. Force measurement of the injured biceps muscle was assessed using the MRC motor scale. Pain at rest was scored using a visual analogue scale (VAS) (0 - no pain to 10 - extreme pain). Finally, the Disabilities of the Arm, Shoulder, And Hand (DASH) questionnaire and the Short-Form Health Survey (SF-) 36 were used to measure quality of life and functional outcome [30, 31]. To compare the DASH score with the SF-36 we calibrated the score to a 100-point metric score from 0 (worst health) to 100 (best health). The SF-36 score ranges from 0 (worst health) to 100 (best health).

CT scan

Patients were scanned using the Aquilion 64-slice CT scanner (Toshiba Medical Systems, Otawara, Japan). A total of 50 consecutive 1.0-mm multiplane reconstruction (MPR) images were computed in the coronal plane perpendicular to the main axis of the humerus. The MPR images were analysed and the mean muscle density (MMD) was calculated [32]. The MMD of the injured (injected) biceps muscle was compared with control muscles (the injured (non-injected) triceps muscle and non-injured (non-injected) contralateral biceps muscle). Further details of the methods are provided as supplementary material.

Quantitative needle EMG

Quantitative needle EMG was performed using disposable EMG needle electrodes with a Medelec Synergy EMG system (Oxford Instruments, Oxford, United Kingdom). EMG of the injured biceps muscle was compared with that of control muscles (the injured (non-injected) brachialis muscle and non-injured (non-injected) contralateral

biceps muscle). Since the mean number of MUPs of the triceps muscle was too low (≤ 2), the brachialis muscle was selected as a control. The multi-MUP analysis was performed using Medelec Synergy software version 11 (Oxford Instruments) [33]. Further details of the methods are provided as supplementary material.

Histochemistry and immunohistochemistry of muscle biopsy

A biopsy of 0.5 cm × 0.5 cm × 0.5 cm of the injured biceps muscle was taken at the site of the MNC injection [34]. It was immediately fixed in 4 % buffered formalin and embedded in paraffin. Tissue sections of 5 µm thickness were cut, deparaffinised and rehydrated for Masson's trichrome staining, routine haematoxylin and eosin (H&E) staining and immunohistochemistry to evaluate muscle, vascular and neural regeneration. For immunohistochemistry Pax7, CD56 and von Willebrand Factor (vWF) were used. Further details of the methods are provided as supplementary material.

Muscle analysis

Muscle sections were analysed using a Zeiss image analysis system (KS400; Zeiss, Sliedrecht, The Netherlands) coupled to a camera (DXC-950P; Sony, Amsterdam, The Netherlands) and ImageJ software v1.38X (National Institutes of Health, Bethesda, Maryland). From each stained section, random images were selected, analysing at least 200 fibres. Further details of the methods are provided as supplementary material.

Statistical analysis

All tests were two-tailed and p - values < 0.05 were considered significant. SPSS v20.0 (SPSS Inc., Chicago, Illinois) was used to perform the analyses. Baseline characteristics are presented as mean and range. Data from muscle biopsies, EMG, CT, and clinical function are presented as mean and standard deviation (sd). Elbow flexion strength using the MRC motor scale is displayed as median and range. A paired *t*-test or one-way analysis of variance (ANOVA) was used to analyse the muscle biopsy parameters. A mixed model analysis was used to analyse EMG, CT and clinical functionality parameters. Patients were included as random effects on each outcome parameter. In order to determine changes in outcome at follow-up, the variable 'time' was included as fixed effect. In order to determine any dose–effect response between group A, B and C, the variable 'group' was also included in the mixed model as a fixed effect.

RESULTS

Baseline patient and injected MNC characteristics are shown in table I. During hospital stay and follow-up, no adverse events in vital signs (blood pressure, pulse

rate, temperature), BM aspiration site, injection sites or surgical wound (haematoma, infection) were observed. Post-operatively, the mean decrease in haemoglobin (Hb) concentration was 0.2 mmol/l (group A), 1.0 mmol/l (group B), and 1.8 mmol/l (group C). Hb concentration was normalized in all patients six months after BM aspiration. In order to assess the safety of MNC injection at a local muscular level, quantitative CT analysis and histology were used. With quantitative CT analysis, the MMD of the injured biceps muscle was compared with injured and non-injured control muscles. A significant decrease in the MMD of the injured biceps muscles in the total patient group was observed ($p = 0.03$). The decrease was 32 % at three months ($p = 0.04$) and 48 % at six months follow-up ($p = 0.009$). No dose effect was observed. The non-injured contralateral biceps and the injured (non-injected) triceps muscle showed no significant change in MMD (figure 1).

A Masson's Trichrome staining was performed to analyse the possible formation of muscle fibrosis after cell injection. As compared with the pre-injection biopsy, a decrease of 52 % in mean areas of fibrosis of the injured biceps muscle was measured at three months after cell injection in the total patient group ($p = 0.01$). No dose effect was observed (figure 2).

Table I: Baseline patient and injected mononuclear cell characteristics

	Group A (n = 3)	Group B (n = 3)	Group C (n = 3)
Age * (years)	33 (20 to 45)	30 (25 to 33)	23 (20 to 28)
Male † (n)	3 (100)	3 (100)	3 (100)
Brachial plexus injury type † (n)			
C5 - C6	1 (33)	1 (33)	0
C5 - C7	0	2 (67)	3 (100)
C5 - Th1	2 (67)	0	0
Time after trauma * (years)	7.4 (2 to 17)	8.0 (3 to 17)	2.9 (2 to 3)
Neurosurgery † (n)	3 (100)	2 (67)	3 (100)
Time after neurosurgery * (years)	7.0 (2.0 to 16.5)	10.1 (4.0 to 16.3)	2.6 (2.2 to 2.9)
Elbow flexion strength # (MRC scale)	3 (3)	2 (2 to 3)	2 (2 to 3)
Active elbow flexion RoM * (°)	65 (25 to 100)	12 (0 to 35)	30 (0 to 90)
Injected mononuclear cells * (x 10^8 cells)	0.89 (0.6 to 1.3)	4 ‡	8 ‡
$CD34^+$ * (x 10^6 cells)	2.5 (0.5 to 5)	10.8 (7 to 16)	23.6 (15 to 32)
$CD34^-$ * (x 10^6 cells)	86.5 (57 to 127)	389 (384 to 393)	776 (769 to 785)

*Values are given as mean with range. †Values are given as number of patients with percentage. #Values are given as median with range. ‡No range is given as the exact number of cells was injected. MRC: Medical Research Council, RoM: range of motion.

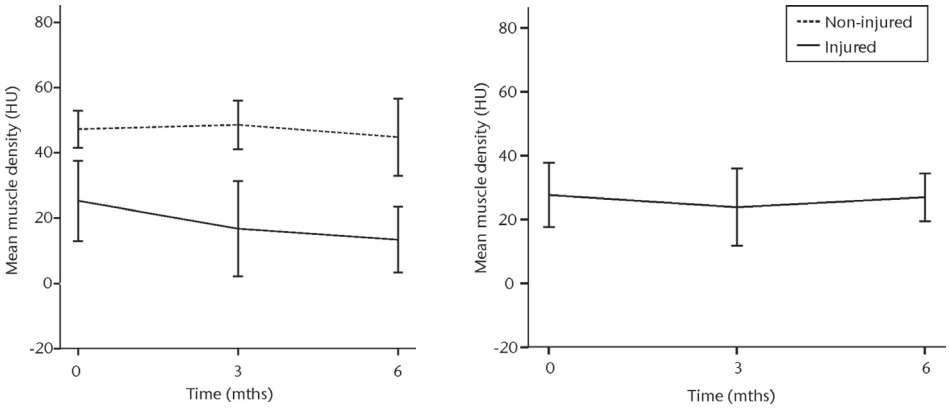

Figure 1: Muscle density
Graphs showing the mean muscle density (MMD) of the injured and non-injured biceps (A) and triceps (B) muscle pre-transplantation and at three and six months follow-up for the total patient group. Error bars denote 95 % confidence interval (HU, Hounsfield unit).

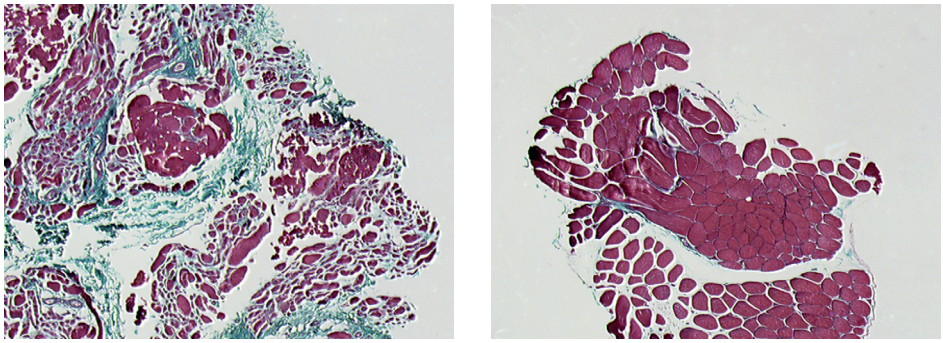

Figure 2: Masson's Trichrome staining
Histological images showing Masson's Trichrome staining of a representative muscle biopsy a) pre- and b) three months post-transplantation (magnification 5 ×).

In order to assess any regenerative potential of the injected MNCs, quantitative needle EMG and histology were performed. The mean amplitude, duration, and number of phases of the MUPs of the injured biceps muscle were compared with injured and non-injured control muscles. After three months, the increase was 36 % in mean amplitude (p = 0.045), 22 % in duration (p = 0.005) and 29 % in number of phases (p = 0.002). The increase in number of phases were sustained at six months follow-up (p = 0.001). A dose-effect relation between group A, B and C was observed only in the mean amplitude (p = 0.03). The non-injured contralateral biceps muscle, the injured (non-injected) brachialis muscle and non-

injured brachialis muscle showed no significant changes in amplitude, duration, or number of MUP phases. The mean number of analyzed MUPs was 5.3 (SD 2.2). This low number represents the severity of the nerve lesions in which patients were unable to recruit more motor units (figure 3).

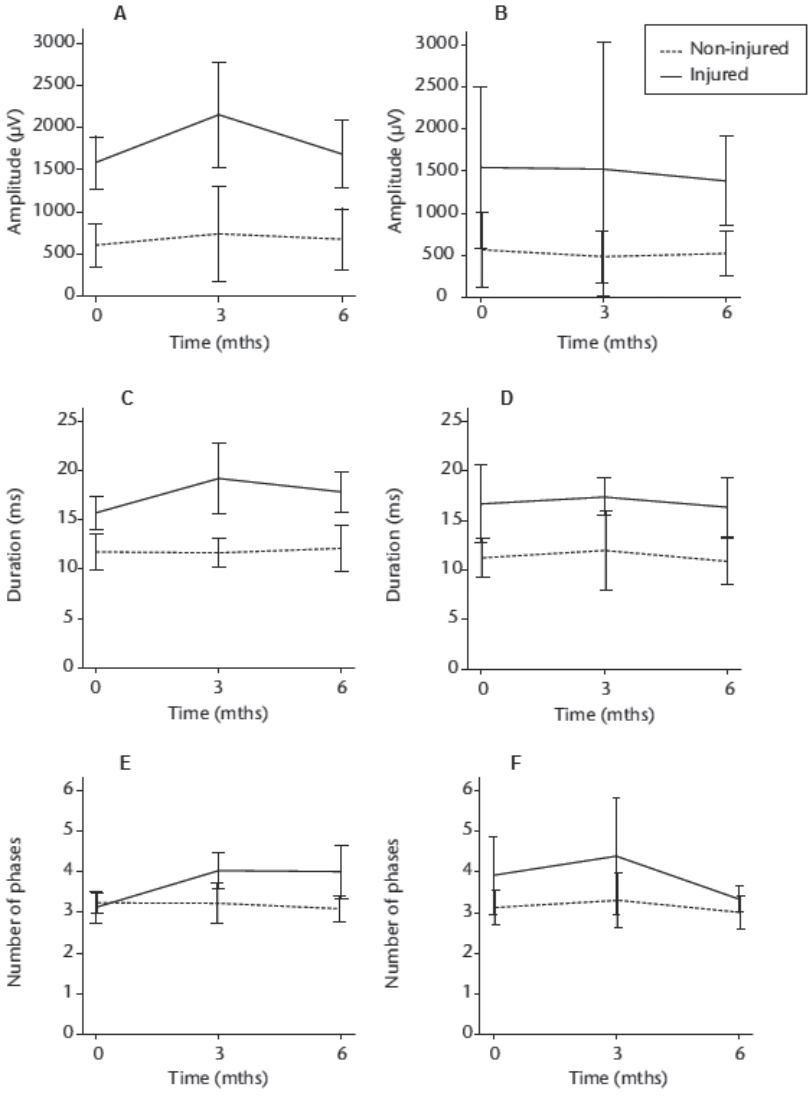

Figure 3: Quantitative needle electromyography
Graphs showing the mean amplitude (a and b), duration (c and d) and number of phases (e and f) of motor unit potentials of the injured and non-injured biceps (left column) and brachialis muscle (right column) pre-transplantation and at three and six months follow-up for the total patient group. Error bars denote 95 % confidence interval.

The myogenic repair potential of the injected MNC cells was also evaluated using the histology of the injured biceps muscle. Analysis of all patients showed a mean increase of 80 % in myofibre diameter three months after the cell injection compared with the pre-injection biopsy ($p = 0.007$). Pax7 staining demonstrated a 50 % increase in the number of satellite cells ($p = 0.045$). Von Willebrand Factor staining showed an increase of 83 % in the number of capillaries per myofibre ($p < 0.001$). No significant increase was demonstrated in the number of centronucleated myofibres or CD56 positive myofibres after cell therapy (table II).

Histological muscle regeneration was most apparent in group B, which observed a 126 % increase in myofibre diameter, a 186 % increase in percentage of centronucleated myofibres, a 117 % increase in number of satellite cells, a 100 % increase in capillaries per myofibre and a 70 % decrease in the area of fibrosis. No dose-effect relation was observed in any of the histological measurements. Figure 4 shows a representative example of H&E, Pax7, and vWF and CD56 staining of a muscle biopsy before and after cell therapy in group B. After cell therapy, the muscle biopsy of one patient in group A did not contain 200 muscle fibres and was therefore excluded from analysis. In group A, no analysis of the CD56 staining could be performed because one patient's muscle biopsy after cell therapy contained only 120 myofibres and was therefore excluded.

In order to observe function improvement after MNC injection, we measured the active flexion ROM and strength of the injured elbow before and after cell therapy compared with the injured and non-injured control muscles. In addition, quality of life questionnaires (SF-36 and DASH) and the VAS score for pain were used. Overall, all patients had a significant increase in active flexion ROM and flexion strength. The flexion ROM increase was 152 % at three months ($p < 0.001$) and 147 % at six months follow-up ($p < 0.001$). The flexion strength increase was 38 % at three months ($p < 0.001$) and 46 % at six months follow-up ($p < 0.001$). As expected, the increase in active elbow flexion ROM and flexion strength was most prominently significant in patient groups where cell therapy was combined with a modified Steindler procedure (groups B and C). No significant changes in SF-36, DASH or VAS score were observed comparing pre- and post-injection at three months (table III).

Table II: Histological results

	Group				
Mean (sd) biceps histology	A (n = 3)	B (n = 3)	C (n = 3)	p-value*	Total (n = 9)
Myofibre diameter (µm)					
0 months	254 (68)	173 (46)	171 (12)		193 (53)
3 months	392 (165)	391 (44)	274 (71)		347 (98)
Mean difference	+138	+218	+103	0.39	0.007 †
Centro-nuclear myofibres (%)					
0 months	5.1 (2.5)	2.1 (1.5)	2.6 (1.7)		3.1 (2.0)
3 months	2.7 (0.8)	6.0 (3.1)	3.4 (1.9)		4.2 (2.5)
Mean difference	-2.4	+3.9	+0.8	0.01	0.30 †
Fibrosis (%)					
0 months	18 (2)	31 (2)	23 (4)		25 (6)
3 months	17 (15)	9 (4)	12 (2)		12 (7)
Mean difference	-1	-22	-11	0.06	0.01 †
Pax7+ myofibres (%)					
0 months	17 (4)	6 (3)	14 (6)		12 (6)
3 months	18 (8)	13 (3)	23 (10)		18 (8)
Mean difference	+1	+7	+9	0.45	0.045 †
vWF+ per myofibre (%)					
0 months	0.8 (0.04)	0.5 (0.1)	0.5 (0.1)		0.6 (0.2)
3 months	1.1 (0.2)	1.0 (0.2)	1.1 (0.4)		1.1 (0.2)
Mean difference	+0.3	+0.5	+0.6	0.33	< 0.001 †
CD56+ myofibres (%)					
0 months	16 (n/a)‡	18 (6)	25 (3)		21 (5)§
3 months	16 (n/a)‡	18 (8)	21 (12)		19 (7)§
Mean difference	0	0	-4	0.94	0.60 †

Mean myofibre diameter, centro-nuclear myofibres, fibrosis, Pax7+ myofibres, vWF per myofibre and CD56+ myofibres of muscle biopsies of the injured biceps muscle before MNC injection and at three months follow-up * comparison of mean differences between time points between the groups (one-way analysis of variance (ANOVA)), † difference between time points for all patients (paired t-test), ‡ standard deviation not available as only one observation was made, § for total seven patients

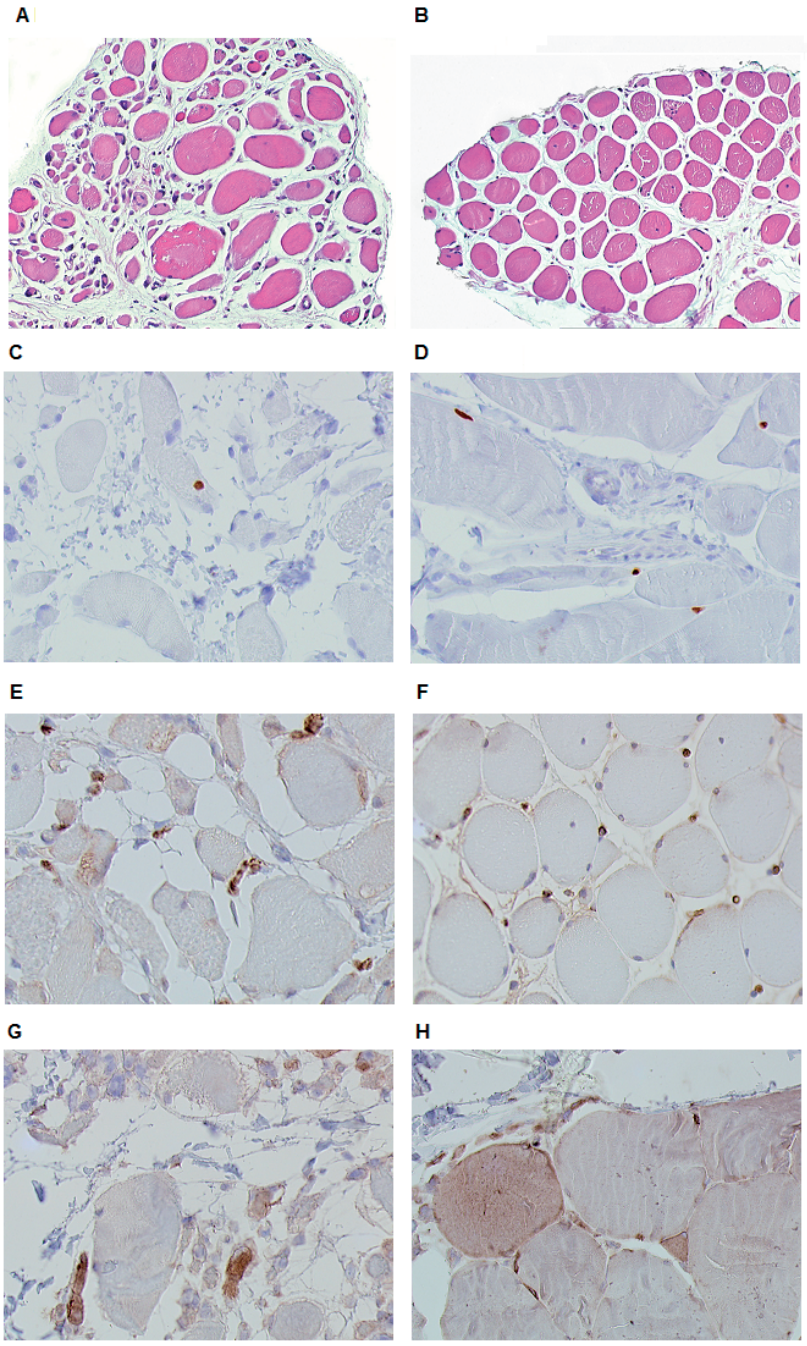

Figure 4: Histology
Histological images for specimens taken pre-transplantation (left column) and at three months follow-up (right column), showing staining of a representative muscle biopsy with haematoxylin and eosin (a and b: × 20), Pax7 (c and d: × 40), vWF (e and f: × 40) and CD56 (g and h: × 40).

Table III: Clinical results

	Group				
Mean (sd) outcome*	A (n = 3)	B (n = 3)	C (n = 3)	*P* - value†	Total (n = 9)
Flexion ROM (°)				0.22	
0 months	65 (38)	12 (20)	30 (52)		36 (41)
3 months	82 (28)	115 (5)	107 (15)		101 (22)
6 months	88 (20)	100 (20)	110 (22)		99 (20)
p-value‡					< 0.001‡
Median flexion strength (MRC grade) (range)				0.02	
0 months	3 (3 to 3)	2 (2 to 3)	2 (2 to 3)		2 (2 to 3)
3 months	3 (3 to 3)	4 (3 to 4)	4 (3 to 4)		3 (3 to 4)
6 monthss	3 (3 to 4)	4 (4 to 4)	4 (4 to 4)		4 (3 to 4)
p-value‡					< 0.001‡
SF-36				0.06	
0 months	86 (6)	57 (16)	69 (6)		78 (15)
3 months	91 (5)	71 (18)	74 (9)		78 (14)
6 months	82 (11)	72 (15)	75 (10)		76 (12)
p-value‡					0.06‡
DASH-DLV				0.15	
0 months	43 (19)	35 (25)	75 (16)		51 (25)
3 months	64 (39)	68 (19)	76 (12)		69 (23)
6 months	41 (16)	71 (17)	74 (16)		65 (20)
p-value‡					0.09‡
VAS for pain				0.02	
0 months	0.8 (0.8)	6.1 (2.1)	1.0 (1.1)		2.7 (2.9)
3 months	0.8 (0.6)	2.3 (2.0)	1.3 (1.6)		1.5 (1.5)
6 months	0.8 (0.6)	2.0 (0.5)	2.2 (3.0)		1.7 (1.7)
p-value‡					0.24‡

Elbow flexion range of movement (ROM) and strength of the injured elbow together with the Short-Form 36 and Disabilities of the Arm Shoulder and Hand (DASH; Dutch language version) questionnaires and visual analogue scale (VAS) for pain before mononuclear cell (MNC) injection and after three and six months * MRC, Medical Research Council; SF-36, Short-Form 36 (scored from 0 to 100); DASH-DLV, Disabilities of the Arm Shoulder and Hand – Dutch language version (scored from 0 to 100); VAS, visual analogue scale (scored from 0 (no pain) to 10 (extreme pain), † comparison of differences between pre-op and six months in the groups (mixed model analysis), ‡ difference between pre-op and six month values for all patients (mixed model analysis)

DISCUSSION

Autologous BM-derived MNC injection in a partially denervated biceps muscle is safe and shows no adverse events with respect to vital signs, BM aspiration sites, injection sites or surgical wounds. Only a mild anaemia was observed, which recovered fully. The interstitial fibrosis present at long-term denervation decreased by 52 % after MNC injection. These results run parallel to the results of CT scan analysis, which demonstrated a decrease of 48 % in MMD.

Our secondary aim was to estimate myogenic repair and reinnervation of partial denervated muscles after MNC injection. Histological analysis, quantitative needle EMG and CT scan analysis demonstrated muscle improvement after MNC injection, which was not present in the control muscles of the sound arm or in the non-injected injured muscles of the affected arm.

Our study has some limitations. While useful to quantify fatty degeneration of rotator cuff muscles, CT analysis only provides an indirect measure of the amount of muscle lipid or fibrosis [32]. We observed a significant decrease in MMD of the injured biceps muscles after cell injection, which could reflect decrease in muscle fibrosis and increase in muscle vascularisation, muscle fattening or oedema from the procedure [35]. Histology of the muscle biopsies demonstrated a 52 % decrease in muscle fibrosis. In addition, an 83% increase in capillaries per myofibre was observed. Using routine H&E staining, no increase in muscle fattening was seen, thus the decrease in MMD reflects either a decrease in muscle fibrosis or an increase in muscle vascularisation. The presence of muscle denervation was assessed using quantitative needle EMG. Analysing a minimum of 20 MUPs per analysed muscle is generally accepted in the literature [33, 36, 37]. Unfortunately, we could not obtain a minimum of 20 MUPs per analysed muscle (mean 5.3 MUPs, SD 2.2), due to the partial denervation. Nevertheless a significant difference in mean amplitude, duration and the number of phases was found.

Another limitation was that in groups B and C, the BM-derived MNC injection was combined with a flexor-pronator transfer, which improves elbow flexion. Since the flexor-pronator group has to be fixated to the distal humerus with a bicortical screw, it is possible that BM may leak underneath the screw, stimulating the muscle in its vicinity (biceps triceps). However, CT changes were not seen in the control triceps muscle, nor were EMG improvements in the injured but non-injected brachialis muscle. A final limitation was low number of patients and lack of a control patient group (i.e. modified Steindler in a partially denervated biceps muscle with no MNC injection).

Quantitative needle EMG was used to determine muscle innervation. After injection of BM-derived MNC cells, an increase of 36 % in amplitude, 22 % in duration, and 29 % in number of phases of MUPs was observed, suggestive for

muscle reinnervation. Both human and animal studies have shown that BM stromal cells can differentiate into neural cells and improve nerve regeneration [38-41].

Histology was used to evaluate the myogenic repair on a microscopic level. Upon muscle denervation, animal studies have shown a decrease from 2104 µm to 50 µm in myofibre diameter, a decrease from 1.6 % to 0.2 % in capillary-to-myofibre ratio and an increase from 11 % to 35 % in fibrosis [14, 17, 18]. Our data are consistent with these studies, demonstrating a mean myofibre diameter of 193 µm, a mean capillary-to-myofibre ratio of 0.6 and a mean fibrosis of 25 % before cell injection.

The number of cells in the satellite pool within a muscle appears to be correlated to postnatal growth characteristics of the muscle (Pax7). In adult muscle, up to 10 % Pax7 positive myofibres have been reported [16, 17, 42]. Before cell injection, we observed mean Pax7 positive myofibres of 11.9 % (SD 6.5). This higher percentage of Pax7 fibres is probably due to the partially denervated biceps muscle in the current study.

After MNC injection, we observed an increase of 50 % in Pax7 positive myofibres and an increase of 83 % in vWF positive capillaries per myofibre. This may demonstrate that MNC injection can result in restoration of the satellite cell pool and vascular bed, which can play an important role in functional recovery following long-term denervation. Indeed, the increase of 80 % in myofibre diameter suggested myofibre regeneration and thus restoration of muscle atrophy. Muscle regeneration was most prominently observed in patient group B, with an increase of 117 % in Pax7 positive myofibres, 100 % in capillary-to-myofibre ratio, 126 % in myofibre diameter, 186 % in centronuclear myofibres and a decrease of 70 % in fibrosis. These results are suggestive of an optimal dose in group B, although a dose-effect response was not significant.

Skeletal muscle is a regenerative tissue in which mononuclear precursor cells respond to injury by dividing and fusing with damaged fibres [15]. As such, it is an attractive target for cell-based therapy. Muscle fibre regeneration levels of up to 12 % have been achieved by using appropriate transplant variables, including cell dose and mode of cell delivery [24]. The present study is the first in which BM transplantation in a human muscle denervation model was assessed with objective measurements (histology, CT muscle and EMG). In humans, transplantation of the MNC fraction of the adult BM enhanced cardiac- and skeletal muscle function in post-ischaemic heart failure and peripheral artery disease [25-28]. These effects could be attributed to the incorporation of stem cells and building muscle or by supplying the logistics required for efficient proliferation of innate cells via paracrine effects [43, 44]. Heterochronic parabiosis was shown to restore the regenerative potential of satellite cells, which suggests that satellite cell activity can be modulated by their microenvironment [45]. Chronic inflammation does not affect

the *in vitro* regenerative potential of human satellite cells, which underscores the *in vivo* influence of the microenvironment on muscle regeneration [46]. Unravelling the molecular mechanism behind the observed regeneration in BPI patients is a challenge that still has to be met. In the future, this can be studied in newly developed animal models representative for BPI [47, 48].

In conclusion, BM-derived MNC injection is safe in partially denervated muscle of traumatic BPI patients. Significant muscle improvement has been observed in muscle biopsies, quantitative needle EMG and CT scan analysis. Although promising, the preliminary results of the present study require confirmation in a larger controlled clinical study.

SUPPLEMENTARY MATERIAL

Bone marrow aspiration and mononuclear cell (MNC) separation

Bone marrow (BM) was aspirated from one location at the posterior iliac crest under aseptic conditions in the operating theatre using a 14-gauge, 40 mm needle (Sterylab, Milan, Italy). The procedure was performed under local (group A) or under general anaesthesia (groups B and C). In group A, 60 ml of BM was aspirated, which was considered the maximum amount that can be aspirated under local anesthesia. Following separation, a mean mononuclear cell (MNC) dose of 0.89×10^8 MNCs (SD 0.38) was obtained. In groups B and C, a MNC dose of 4.0×10^8 MNCs and 8.0×10^8 MNCs was anticipated, respectively. In order to get these cell concentrates, 350 ml and 650 ml of BM was aspirated under general anaesthesia for groups B and C, respectively. Due to ethical considerations, in these two patient groups BM aspiration was performed in combination with a modified Steindler procedure, which is our standard secondary surgical procedure with a flexion deficiency of the elbow. BM was collected in flasks containing Hanks balanced salt solution and heparin and was then separated over a density gradient (Ficoll isopaque specific density of 1.077 g/cm^3). The MNCs were harvested and washed in 0.9 % NaCl with 0.5 % human serum albumin (RVG 16910; Sanquin, Amsterdam, The Netherlands). The BM-derived MNC doses were concentrated in a volume of 10 ml. The Ficoll isolation procedure was performed in a clean-room facility according to good manufacturing practice (GMP) and took approximately three hours [25, 28].

MNC injection

BM-derived MNCs were injected in the biceps brachii muscle. The 20 injections were centred on the largest diameter of the muscle belly at a distance of approximately 0.5 cm × 0.5 cm from each other. With each injection, a volume of 0.5 ml MNCs was injected at a standard injection depth in the muscle belly (which was palpated manually) of 0.5 cm using a 26-gauge needle (Becton Dickinson,

Breda, the Netherlands). The same surgeon, who also performed the tendon transfer, performed these injections (RN).

Clinical functionality

In order to maximise reliability and validity of the range of movement (ROM) measurements, the arm was kept in a standardised position (0 ° anteflexion, 0 ° abduction, 0° rotation of the shoulder). Force measurement of the injured biceps muscle was also performed in a standardised arm position (0 ° anteflexion, 0 ° abduction, 0 ° rotation of the shoulder and 90 ° elbow flexion). All measurements were performed by the same examiner (RN).

CT scan

The Aquilion 64-slice CT scanner was used (Toshiba Medical Systems, Otawara, Japan). The scanning parameters were set at 135 kVp and a detector pitch of 53 (pitch factor 0.828). A soft-tissue filter and boost three-dimensional (3D) artifact suppression was used, producing a 512 × 512 matrix of 1 mm thick slices (slice overlap: 0.2 mm). A total of 50 consecutive multiplane reconstruction (MPR) images were computed by reconstructing images in the coronal and sagital plane so that in every patient axial, slices were used for analysis, thereby correcting for oblique slices. As observed, the largest transversal diameter of the biceps brachii muscle was shifted from the midpoint of the humerus to the distal 2/3 point of the humerus. Therefore, 50 consecutive images were selected from 25 mm superior and 25 mm inferior to this distal 2/3 point of the humerus. This was measured using the most superior part of the humeral head and the most superior part of the olecranon. Images were first recalibrated for air (-1000 HU) based on samples outside the patient. Subsequently, the triceps muscle and the biceps brachii muscle were manually outlined as separate regions of interest (ROI). All pixels containing bone tissue were automatically excluded from the segmentation by applying a threshold value of 200 HU. A histogram was constructed from all pixels within the outlined ROI to calculate the mean muscle density (MMD) [32].

Quantitative needle electromyography

Quantitative needle electromyography (EMG) was performed using the Medelec Synergy EMG system (Oxford Instruments, Oxford, United Kingdom). The EMG signals were recorded with filter settings of 5 Hz for high pass and 10 kHz for low pass. For the biceps brachii muscle, the electrode was positioned in the middle of the muscle belly. For the brachialis muscle, the electrode was positioned medial to the biceps muscle at the level where the latter muscle adheres to the tendon. When the EMG signal was crisp, no further adjustment of the electrode position was made and motor unit potentials (MUPs) during voluntary contraction were recorded

for 30 seconds. Potentials that were regarded as belonging to unique motor units but were judged to represent a motor unit already collected were omitted, as well as those with a noisy baseline or other artifacts. Among the remaining MUPs, the automatic duration cursor settings were manually corrected if necessary. If the duration cursor was changed, the amplitude and number of phases of the MUPs were recalculated before analysis [33]. All analyses and re-analyses were performed by the first author (SH) and an experienced neurophysiologist (JvD) blinded to the patient characteristics.

Histochemistry and immunohistochemistry of muscle biopsy

A biopsy of the injured biceps muscle was performed at the site of the MNC injection using a forceps with two sharp-edged jaws (Blakesley Conchotoma; DK Instruments, West Bengal, India) [34]. The muscle biopsies before and after cell therapy in group A were taken under local anaesthesia at the outpatient clinic. The muscle biopsies before cell therapy in groups B and C were performed under general anaesthesia in combination with the modified Steindler procedure. The muscle biopsies after cell therapy in groups B and C were taken under local anaesthesia at the outpatient clinic.

For Masson's trichrome staining, sections were incubated subsequently for 60 minutes at 56 °C in Bouin's fluid (640960; Klinipath, Duiven, the Netherlands), 15 minutes in 1 % Biebrich Scarlet (03336; Brunschwig Chemie, Amsterdam, the Netherlands), 15 minutes in 5 % Tungstophosphoric acid (1005830100; Merck, Damstadt, Germany) and 10 minutes in 2 % light green SF yellowish (C.I. 42095, 115941; Merck) resulting in a green staining for fibrosis and red staining for muscle fibres.

For immunohistochemistry, the primary antibodies monoclonal mouse anti-human Pax7 (1:20, 352-523; Developmental Studies Hybridoma Bank, Iowa City, Iowa), monoclonal mouse anti-human CD56 (1:80, clone NCAM16.2, 345811; BD Biosciences, Erembodegem-Aalst, Belgium), and polyclonal rabbit anti-human von Willebrand Factor (vWF) (1:4000, A0082; Dakocytomation, Heverlee, Belgium) were used. Sections were pretreated by boiling for 20 minutes at pH 6.0 in 10 mM citric acid buffer (C0759-500G; Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) (Pax7 and vWF)or at pH 9.0 in 1 mM ethylenediamine-tetraacetic acid disodium salt buffer (1073; Baker, Deventer, the Netherlands) (CD56) for antigen retrieval. Next, sections were incubated for 60 min in 5 % normal goat serum (NGS) (X0907; DakoCytomation, Heverlee, Belgium) and 1 % bovine serum albumin (BSA) (A9647-50G; Sigma-Aldrich Chemie) in phosphate buffered saline (PBS) to block nonspecific binding. For Pax7, 0.3 % Triton X 100 (T8787; Sigma-Aldrich Chemie) and 0.01 % hydrochloric acid (1003171000; Merck) was

added to the blocking solution. Sections were incubated with the primary antibodies overnight and subsequently for 20 minutes in 0.3 % hydrogen peroxide (1072090250; Merck) in PBS to block endogenous peroxidase. Next, the sections were incubated for 60 minutes with the biotinylated secondary antibody rabbit anti-mouse (1:200, E0464; Dako-Cytomation) (Pax7, CD56) or goat anti-rabbit (1:200, E0432; DakoCytomation) (vWF). In order to visualise immunolabelling, sections were incubated for 30 minutes with horseradish peroxidase labeled avidinbiotin complex (Vectastain Elite ABC kit, PK6100; Brunschwig, Amsterdam, the Netherlands) and subsequently with diaminobenidine (DAB liquid, K3468; DakoCytomation) as chromogenic substrate resulting in a brown precipitate. Finally, sections were counterstained for one minute with Harris' haematoxilline solution (1092532500; Merck), dehydrated and mounted on Micromount (1731; Surgipath Europe, Peterborough, United Kingdom).

Muscle analysis

The area of fibrosis was analysed in the Masson-trichrome stained sections by dividing each pixel into three color components (hue, saturation, intensity). The threshold was defined and kept constant throughout the analysis. The percentage of green fibrotic area was measured. The haematoxylin and eosin (H&E) stained sections were used to measure the myofibre diameter and the percentage of centronucleated, regenerating myofibres. In order to measure the myofibre diameter and the percentage of centronucleated myofibres, only transversely cut myofibres were measured. In order to compensate for myofibres that were partially longitudinal the minimal diameter was calculated. The Pax7 and CD56 stained sections were used to calculate the percentage of myofibres containing a Pax7 positive cell and percentage of CD56 positive myofibres respectively. The vWF sections were used to assess the number of capillaries around each fibre. All transversely cut capillaries were counted. If a capillary was sectioned longitudinally, it was counted as one each time it crossed a junction between three or more muscle fibres.

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ICMJE CONFLICT OF INTEREST

None declared

AUTHOR CONTRIBUTIONS

S. Hogendoorn: Design of study, Data collection, Data analysis, Writing the paper
B. J. Duijnisveld: Data collection, Data analysis, Writing the paper
S. G. van Duinen: Data collection (histology), Data analysis, Writing a part of the paper
B. C. Stoel: Data collection (quantitative CT), Data analysis, Writing a part of the paper
J. G. van Dijk: Performing the EMGs, Data collection (EMG), Data analysis, Writing a part of the paper
W. E. Fibbe: Facilitating with the stem cell laboratory (BM-derived MNC separation), Design of the study, Writing a part of the paper
R. G. H. H. Nelissen: Design of the study, Performing the surgeries, Data collection (clinical studies), Data analysis, Writing the paper

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Chapter 9

Discussion

SUMMARY AND RECOMMENDATIONS FOR FUTURE RESEARCH

Functional level

The aim of this thesis is to evaluate determinants of outcome, which will have an effect on overall functionality of the patient with an OBPI. Symptoms of OBPI vary widely over the course of time and from individual to individual and are related to various degrees of denervation, muscle weakness, contractures and bone deformities with their subsequent functional limitations. To date, no universally accepted overall framework is available to assess the outcome of patients with OBPI. At the functional level, the developmental process of outcome measures using the ICF is described (chapter 2). The first step is to conduct four preparatory studies to identify ICF categories important for OBPI: a) a systematic literature review to identify outcome measures, b) a qualitative study using focus groups, c) an expert survey and d) a cross-sectional, multicentre study. The proposed method to develop ICF Core Sets for OBPI yields a practical tool for multiple purposes: for clinicians to systematically assess and evaluate the individual's functioning, for researchers to design and compare studies, and for patients to get more insight into their health problems and their management.

Clinical level

At the clinical level, impaired muscles and joint deformities and their treatment options are addressed. Long-term denervation results in muscle degeneration including muscle atrophy, fatty degeneration and interstitial fibrosis in the muscle. Assessment of contractile CSA might yield valuable insight in pathophysiology and might be used to predict the outcome of conservative and surgical procedures. Volumes of interest were manually drawn on MRI scans by two independent observers to calculate the percentage of muscle fat, the total CSA and the contractile CSA in both the sound and the injured upper extremity in patients with a traumatic BPI (chapter 3). The contractile CSA showed the highest association with active flexion and muscle force compared to fat percentage and total CSA. The mean contractile CSA was lower in the affected biceps brachii 8 cm^2 (SD 5.1) compared to a mean of 19 cm^2 (SD 4.9) in the non-affected biceps brachii ($p < 0.001$) with an excellent inter-observer reliability (ICC 0.88). The contractile CSA of the affected biceps brachii was associated with active elbow flexion with an estimate of 7.1 °, 95 % CI 2.8 to 11.5, $p = 0.003$), i.e. an increase of 1.0 cm^2 of contractile CSA results in an increase of 7.1 ° active elbow flexion. The contractile CSA of the affected biceps brachii was associated with muscle strength in MRC with an odds ratio of 2.6 (95 % CI 1.1 to 6.1, $p = 0.029$). One cm^2 increase in contractile CSA of the affected biceps brachii was associated with an increase of 13 Newton muscle force (95 % CI 8.9 to 17.1, $p < 0.001$).

The effect of serial casting on elbow flexion contractures in OBPI was evaluated (chapter 4). A prospective consecutive cohort study was performed with a median of 5-year follow-up. Forty-one patients with elbow flexion contractures ≥ 30° were treated with serial casting for a maximum of 8 weeks until the flexion contracture was ≤ 10 °. Serial casting improved elbow flexion contractures with an increase of passive extension from a median -40 ° (IQR -50 to -30) before casting to -15 ° (IQR -10 to -20, $p < 0.001$) after casting. Recurrences were frequently observed. Overall, twenty (49 %) patients showed thirty-seven recurrences. The severity of elbow flexion contracture was only a small predictor for recurrent elbow flexion contracture with a hazard ratio of 0.93 (95 % CI 0.89 to 0.96, $p < 0.001$).

The effect of botulinum toxin A (BTX-A) injection in the subscapular muscle in patients with internal rotation contracture was evaluated using the external rotation and the need for tendon transfer for external rotation as outcome (chapter 5). A prospective comparative study was performed including 15 consecutive patients treated with BTX-A (2 IU/kg body weight) under general anaesthesia and a historic control group of 67 patients with mean age 30 months (SD 10). BTX-A injection in the subscapular muscle increased the passive external rotation in adduction from -1 ° (95 % CI -10 to 8) to 32 ° (95 % CI 17 to 46) at 3 months. BTX-A injection also reduced the need for tendon transfer surgery as 6 patients were indicated for surgery in the BTX-A group compared to 66 in the control group. At 5 years follow-up, 10 patients in the BTX-A group were indicated for surgery with a hazard ratio of 4.0 (95 % CI 1.9 to 8.4).

Cellular level

Finally at the cellular level, muscle degeneration is characterized in both inflammation (rheumatoid-RA) and osteoarthritis (OA) as well as in denervated muscles. In the latter group, also cell therapy is explored as a treatment option. The effect of chronic inflammation compared to non-inflammatory muscles around the knee joint on the regenerative potential of satellite cells in human muscle was studied (chapter 6, 7). As a model for chronic inflammation, muscle biopsies form 16 patients suffering from osteoarthritis were compared to 11 patients suffering from rheumatoid arthritis. Histological characteristics showed no significant differences in type II muscle fiber atrophy, lipofuscin accumulation, or satellite cell number in RA compared to OA patients. After mononuclear cell explantation, myogenic purity, viability, proliferation index, number of colonies, myogenic colonies, growth speed, maximum number of population doublings and fusion index were not different between RA and OA patients. Furthermore, the expression of proteins involved in replicative and stress-induced premature senescence and apoptosis, including p16, p21, p53, hTERT and cleaved caspase-3, was not different

between OA and RA patients. Mean telomere length was shorter in the RA group compared to the OA group. In conclusion, chronic inflammation in RA does not affect the *in vitro* regenerative potential of human satellite cells.

A pilot study is described to assess the safety and regenerative potential of autologous bone marrow-derived mononuclear cell injection in a partially denervated biceps muscle of BPI patients (chapter 8). Nine adult traumatic BPI patients with insufficient active elbow flexion received intramuscular escalating doses of autologous bone marrow-derived mononuclear cells, combined with an elbow flexorpronator group transfer (Steindler procedure). Mononuclear cell injection in a partially denervated biceps muscle of these brachial plexus patients was safe and showed no adverse effects on vital signs, bone marrow aspiration sites, injection sites, or surgical wound. Results suggest enhanced muscle regeneration with a 52 % decrease in muscle fibrosis ($p = 0.01$), an 80 % increase in myofiber diameter ($p = 0.007$), a 50 % increase in satellite cells ($p = 0.045$) and an 83 % increase in capillary-to-myofiber ratio ($p < 0.001$) was shown. CT analysis demonstrated a 48 % decrease in mean muscle density ($p = 0.009$). Motor unit analysis showed a mean increase of 36 % in motor unit amplitude ($p = 0.045$), 22 % increase in duration ($p = 0.005$) and 29 % increase in number of phases ($p = 0.002$).

Recommendations for future research

At the functional level, studies on the evaluation of OBPI show high variability between the used outcome variables. Children with OBPI experience difficulties in all areas of functioning, as well as in both environmental and personal factors [1]. These results underscore the need for the development and use of outcome variables representing all domains of health status in these patients. Future research should include the evaluation of functionality problems in OBPI patients with an expert survey and a cross-sectional, multicentre study. The latter was recently done at our institution in 1000 Patients (ZAP-Plexus www.zenuwcentrum.org). As for the definition of valid functionality outcome scores for OBPI, a first version of ICF Core Sets should be defined at a Delphi conference to integrate the evidence from the preparatory studies. In a second step, field-testing among patients should validate this first version of Core Sets for OBPI. In the future, standardised ICF Core Sets will not only be useful for research, but also in the important shared decision making process between patient and physician. Eventually these ICF data can be used to quantify the severity of OBPI and compare them between patient groups and even medical centers, to improve decision making in the clinical settings.

A result of muscle degeneration includes the development of contractures including elbow flexion contractures and internal rotation contractures around the shoulder, both affecting functionality. Future research should gain more

insight into the underlying factors of contractures including muscle imbalance, reduced longitudinal muscle growth, capsular fibrosis and posture which could reveal possible treatment strategies [2-6]. To unravel the impaired movement, the combination of a haptic manipulator and surface EMG could be use to discriminate neural (active and reflexive) and non-neural contributors (passive muscle and connective tissue properties) [7]. Muscle degeneration as reflected by both muscle atrophy and fatty degeneration can be quantified using three-point Dixon MRI with an excellent reproducibility. Contractile CSA was significantly associated with active muscle flexion force. Future research should determine whether quantitative measurement of muscle degeneration could predict the outcome of conservative and surgical procedures. Case reports on posterior shoulder dislocation at young age have been described before, suggesting direct trauma at birth as a cause of the dislocation [8-11], which is contradictory to observations at successive MRI's in our clinic [12]. However, at outdoor clinics the awareness of the occurrence of potential contractures should be recognised at the shoulder (internal rotation and abduction contractures) and at the elbow (flexion and supination contractures). Therefore, specialised multidisciplinary clinics (the Dutch model), involving neurosurgeons, orthopaedic surgeons, rehabilitation physicians as well as physiotherapists and ergo therapists are necessary for optimal care of the patients and their family.

At the cellular level, muscle regeneration has potential despite the partial denervation [13, 14]. Cell therapy can be a therapeutic option to replenish the exhausted satellite cell pool. Future research should focus on the identification of mechanisms influencing muscle regeneration by modulation of its microenvironment to improve strategies for muscle regeneration. Furthermore, future research should determine whether a combination of nerve repair en muscle regeneration could be effective. Animal models are available to identify mechanisms for nerve repair and muscle regeneration in BPI [5, 15, 16].

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Chapter 10

Dutch summary

NEDERLANDSE SAMENVATTING

Plexus brachialis letsel

Het plexus brachialis letsel is een tractie letsel van de plexus brachialis wat kan ontstaan tijdens de partus of door een trauma op latere leeftijd. Er is een ruime variëteit in het type symptomen waar patiënten met een plexus brachialis last van kunnen ondervinden zoals verschillende mate van denervatie, spierzwakte, contracturen, botdeformiteiten en functionele beperkingen. Ondanks natuurlijk herstel is er vaak neurochirurgische en orthopaedisch chirurgische behandeling noodzakelijk om de functie van de schouder, elleboog, pols en hand te verbeteren. Om te bepalen of een behandeling nodig is en om te onderzoeken wat het resultaat is van een behandeling, is het noodzakelijk om een wereldwijd geaccepteerde en betrouwbare methode te ontwikkelen om de mate van herstel na het plexus brachialis letsel te kunnen beschrijven (hoofdstuk 2). De eerste stap hiervoor is het verrichten van 4 voorbereidende onderzoeken: een systematische literatuurstudie naar welke meetinstrumenten gebruikt worden, een qualitatieve studie naar symptomen van een plexus letsel. Dit wordt gedaan met patiënt focusgroepen, een enquête onder deskundigen en een zogenaamde cross-sectionele studie van deze patiënten. Met de resultaten van deze 4 voorbereidende onderzoeken wordt een eerste versie van kernsymptomen geformuleerd tijdens een consensus conferentie. Vervolgens wordt deze eerste set van kernsymptomen getest bij patiënten met een plexus brachialis letsel.

Spierdegeneratie

Langdurige denervatie resulteert in spierdegeneratie waaronder atrofie, spiervervetting en fibrose in de spier. Het doel van de studie in hoofdstuk 3 was om de mate van spieratrofie en vervetting van spieren in de bovenarm te meten en te analyseren of spierdegeneratie geassocieerd was aan de mate van beweging van de elleboog en de biceps spierkracht. Het vetpercentage, de totale oppervlakte van de spier en de contractiele oppervlakte van de spier was te meten met een zeer hoge betrouwbaarheid. De contractiele oppervlakte was lager in de aangedane biceps (gemiddeld 8 cm^2) vergeleken met de niet aangedane biceps (gemiddeld 19 cm^2). Uit de multivariate analyse bleek dat de contractiele oppervlakte van de bicepsspier geassocieerd was met de mate van buigen van de elleboog en aan de spierkracht.

Het doel van de studie in hoofdstuk 4 was om te evalueren of gipsredressie effectief is om elleboog flexiecontracturen te behandelen bij patiënten met een obstetrisch plexus brachialis letsel. Een cohort studie werd verricht met 41 patiënten die gedurende 5 jaar werden gevolgd. Deze patiënten hadden een elleboog flexiecontractuur van 30 graden of meer en werden maximaal 8 weken behandeld

met gipsredressie totdat de elleboog flexie contractuur 10 graden of minder was. Gipsredressie verbeterde de passieve elleboog extensie van een mediaan van -40 graden naar -15 graden. Bij 20 patiënten ontstond een recidief waarvoor zij opnieuw behandeld werden met gipsredressie. De ernst van de contractuur was een geringe voorspeller voor het krijgen van een recidief. De patiënttevredenheid was gemiddeld. Bij 4 patiënten ging de flexie achteruit en bij 2 patiënten moest de behandeling met gipsredressie voortijdig vervangen worden door een nachtspalk omdat zij klachten hadden van de gipsredressie.

Het effect van botoxinjectie in de subscapularis spier om endorotatie contracturen te verminderen bij patiënten, werd onderzocht in hoofdstuk 5. Een vergelijkende studie werd verricht met 15 patiënten die behandeld werden met botox en 67 patiënten die in het verleden geen botoxinjectie hadden ondergaan. Na 3 maanden was de passieve exorotatie in adductie in de botoxgroep toegenomen van -1 graden naar 32 graden. Patiënten zonder botoxinjectie hadden een verdere verslechtering van de passieve exorotatie. Botoxinjectie verminderde ook de noodzaak tot een spierpees transpositie. Na 5 jaar hadden 10 patiënten (67 %) in de botoxgroep een operatie-indicatie versus 66 patiënten (99 %) in de groep zonder botox behandeling.

Spierregeneratie

Satellietcellen zijn de stamcellen van een spier en zorgen voor spierregeneratie. Niet alleen de satellietcellen zelf, maar ook omgevingsfactoren kunnen van invloed zijn op de regeneratie capaciteit. Het doel van de studies in de hoofdstukken 6 en 7 was om de invloed van ontstekingsfactoren te onderzoeken op de regeneratiecapaciteit van satellietcellen. Tijdens het plaatsen van een totale knieprothese, werden spierbiopten genomen van 16 patiënten met artrose en deze biopten werden vergeleken met spierbiopten van 11 patiënten met reumatoïde artritis die een hoge mate van ontstekingsfactoren hebben. De spiercelhistologie was niet verschillend tussen patiënten met artrose of reuma. Bij het kweken van de satellietcellen was er geen verschil in de groeisnelheid, levensvatbaarheid en de differentiatiecapaciteit. Ook de expressie van eiwitten die betrokken zijn bij de regeneratiecapaciteit, was niet verschillend tussen beide groepen. De gemiddelde telomeerlengte was echter wel korter in reumapatiënten vergeleken met artrosepatiënten. De conclusie was dat satellietcellen in staat zijn om te delen en te differentiëren indien zij uit hun omgeving met ontstekingsfactoren gehaald worden.

In hoofdstuk 8 werd de veiligheid en het regeneratie potentieel van mononucleaire cellen uit autoloog beenmerg onderzocht. Bij 9 patiënten met een plexus brachialis letsel werden mononucleaire cellen uit het beenmerg gewonnen en geïnjecteerd in de partieel gedenerveerde bicepsspier. Er werden geen bijwerkingen gezien. Spierbiopten lieten 52% minder fibrose zien, 80% toename

in spiervezel diameter, 50% toename in aantal satellietcellen en 83% toename in bloedvaten-spiervezel verhouding. De CT scan liet een afname zien van 48% in spierdichtheid en het electromyogram liet een toename zien van 36% in amplitude, 22% in duur en 29 % in het aantal fases. Deze resultaten zijn allen suggestief voor spier regeneratie na de injectie van mononucleaire cellen uit beenmerg.

Appendices

PUBLICATIONS

- Quantitative Dixon and qualitative T1 MRI sequences to relate muscle atrophy and fatty degeneration with range of motion and muscle force in brachial plexus injury
  B.J. Duijnisveld, J.F. Henseler, M. Fiocco, M. Reijnierse M, H.E. Kan, R.G.H.H. Nelissen, Submitted

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CURRICULUM VITAE

Bouke Duijnisveld was born on the 16th of November 1980 in Garrelsweer, the Netherlands. He graduated from high school in 1999 at the Willem Lodewijk Gymnasium in Groningen. In that same year he started to study biomedical sciences at the Leiden University Medical Center. After his Bachelor of Science graduation in 2002, he started his medical study and combined this with his Master in Biomedical Sciences. During his studies he was a teaching assistant at the department of Neuroanatomy and research assistant at the department of Radiology of the Leiden University Medical Center. He resided in over 20 committees of a variety in nature, all of which was to improve education of to make it a more joyful. He interrupted his studies when he was elected president of the Leiden Medical Students´ Association (M.F.L.S.) in 2004-2005. For the past 10 years, he was board member of the local organization KNMG of Gouda, Alphen aan den Rijn and Leiden. During his clinical rotations he performed his Peadiatrics clerkship at the Yong Loo Lin School of Medicine in Singapore in 2006. He combined clinical work with research during his final year of his studies at the department of Orthopaedics of the Leiden University Medical Center.

At the end of his studies he obtained a ZonMw grant for his research project on muscle and joint sequelae in brachial plexus injury. In 2008 he received both his Medical Degree and Master of Science Degree in Biomedical Sciences. After graduation, he continued his research with the basic science and clinical trials described in this thesis under supervision of prof. dr. R.G.H.H. Nelissen. In 2010 he received a grant from the Dutch Rheumatoid Arthritis Association to perform his studies on human satellite cells under supervision of dr. G. Butler-Browne at Université Pierre et Marie Curie, Centre de Recherche en Myologie, Groupe Hospitalier de la Pitié-Salpêtrière, Paris, France.

In 2011 he commenced his medical training in Orthopaedic Surgery under supervision of prof. dr. R.G.H.H. Nelissen. He performed his General Surgery at the Bronovo hospital in The Hague and continued with Orthopaedic Surgery in the Leiden University Medical Center in Leiden and the Medical Center Haaglanden in The Hague. In 2015 he interrupted his training to finish the research which resulted in this thesis. He presented the results described in this thesis on a number of national and international conferences and published them in peer-reviewed journals. In 2016 he continued with his medical training in Orthopaedic Surgery at the Amphia Hospital in Breda where he will finish his training in 2018.