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MICRONEUROVASCULAR FREE MUSCLE TRANSFER WITH CROSS-OVER NERVE GRAFTS IN FACIAL REANIMATION

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Academic Dissertation

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“ On ne voit bien qu’avec le cœur. L’essentiel est invisible pour les yeux.”

Antoine de Saint-Exupery, Le Petit Prince

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II Ylä-Kotola TM, Kauhanen MSC, Koskinen SK, Asko-Seljavaara SL. Magnetic resonance imaging of microneurovascular free muscle flaps in facial reanimation. Brit J Plast Surg 58:22-27, 2005.

III Kauhanen MSC, Ylä-Kotola TM, Leivo IV, Tukiainen E, Asko-Seljavaara SL. Long-term adaption of human microneurovascular muscle flaps to the paralyzed face. An immunohistochemical study. Microsurgery 26:557-565, 2006.

IV Ylä-Kotola TM, Kauhanen MSC, Asko-Seljavaara SL, Haglund CH, Tukiainen E, Leivo IV. P75 Nerve growth factor receptor is expressed in regenerating human nerve grafts. J Surg Res 146(2):254-61, 2008.

V Ylä-Kotola TM, Kauhanen MSC, Asko-Seljavaara SL, Haglund CH, Tukiainen E, Leivo IV. VEGF and its receptors are expressed in human nerve grafts. Manuscript, submitted.

ABBREVIATIONS AND SYNONYMS

Acoustic neurinoma	vestibular schwannoma
CD-31	cluster of differentiation 31
Flk-1	fetal liver kinase-1, VEGFR-2, KDR
Flt-1	fms-like tyrosine kinase-1, VEGFR-1
KDR	kinase insert domain-containing receptor
Ki-67	nuclear protein expressed in proliferating cells
MNV	microneurovascular
Mnv muscle transfer	functional muscle transfer
MRI	magnetic resonance imaging
NGF	nerve growth factor
NGFR	nerve growth factor receptor
NF-200	neurofilament-200
PECAM-1	platelet endothelial cell adhesion molecule
SMAS	superficial musculoaponeurotic system
Trk	tropomyosin receptor kinase
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

ABSTRACT

Background

Facial expression is an important part of human communication. Loss of facial symmetry and mimetic function after facial nerve injury has an enormous impact on the psychosocial conditions of the patients. Therefore, surgical restoration of the facial nerve and muscles is a great challenge. Patients with severe long-term facial paralysis are often reanimated with a two-stage procedure combining cross-facial nerve grafting, and 6 to 8 months later with microneurovascular (MNV) muscle transfer. Many factors contribute to the functional outcome of reconstruction; some of them are well-known but there are still many questions unanswered.

Material and Methods

In this thesis, we recorded the long-term results of MNV surgery in facial paralysis and observed the possible contributing factors to final functional and aesthetic outcome after the two-stage MNV reanimation procedure. Twenty-seven out of forty patients operated on were interviewed, and the functional outcome was graded on House's scale. Hospital charts were reviewed to collect demographic and clinical data. Fifteen patients were available for the magnetic resonance imaging of MNV muscle flap to describe the survival and volume of these flaps. Nerve graft samples (n=37) were obtained in the second stage of the operation. Muscle biopsy samples (n=18) were taken from MNV muscle flaps during secondary refinement procedures. The structure of MNV muscles and nerve grafts was evaluated using basic histology and immunohistochemical methods. Immunohistochemistry for muscle biopsies revealed muscle fiber type distribution (anti-myosin fast), proliferating satellite cells (Ki-67) and reinnervation indicating the Schwann cells (S-100). Muscle atrophy was assessed histomorphometrically. Immunohistochemistry for nerve graft samples revealed number of viable axons (NF-200), Schwann cells (S-100) and vascular structures (CD-31) within the nerve graft. Immunoreactivity for p75 nerve growth factor receptor (p75NGFR), vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptors (Flt-1, Flk-1) was also characterized in nerve graft samples. Statistical analysis was performed, comparing the data obtained.

Results

1. Two-thirds of the patients achieved good result in facial reanimation defined as mild or moderate dysfunction. The longer the follow-up time after muscle transfer the weaker was the muscle function. A majority of the patients (78%) defined their quality of life improved after surgery.
2. In magnetic resonance images (MRI), the volume of the free MNV flaps declined significantly with the follow-up. A correlation was found between good functional outcome and normal muscle structure in MRI.
3. In muscle biopsies, the mean muscle fiber diameter was diminished to 40% compared to control values. Muscle atrophy was not type specific. Proliferative activity of satellite cells

was seen in 60% of the samples and it tended to decline with an increase of follow-up time. All samples showed intramuscular innervation. Severe muscle atrophy correlated with prolonged intraoperative ischaemia. The good long-term functional outcome correlated with dominance of fast fibers in muscle grafts.

4. In nerve grafts, the mean number of viable axons (NF-200) amounted to 38% of that in control samples. The grafted nerves were characterized by fibrosis and invasion of inflammatory cells, and regenerated axons were thinner than in control samples. A longer time between cross facial nerve grafting and biopsy sampling correlated with a higher number of viable axons. P75NGFR was expressed in every nerve graft sample. The expression of p75NGFR was lower in older than in younger patients. A high expression of p75NGFR was often seen with better function of the transplanted muscle.

5. Density of vascular structures corresponded to that of control nerves. In grafted nerve VEGF, Flt-1 and Flk-1 were expressed in nervous tissue. In control samples, the VEGF expression was low and located mainly in myelin sheaths.

Conclusions

In conclusion, most of the patients achieved good result in facial reanimation and were satisfied with the functional outcome. The mimic function was poorer in patients with longer follow-up time. MRI can be used to evaluate the structure of the microneurovascular muscle flaps. Regeneration of the muscle flaps was still going on many years after the transplantation and reinnervation was seen in all muscle samples. Grafted nerves were characterized by fibrosis and fewer, thinner axons compared to control nerves. All nerve grafts were well vascularized. P75NGFR and VEGF were expressed in human nerve grafts with higher intensity than in control nerves which is described for the first time.

INTRODUCTION

Free muscle flaps provide an important source of tissue when reconstructing tissue defects or functional deficits due to congenital problems or acquired diseases. In free muscle transfers, the muscle is harvested with its arterial and venous vessels and transferred to the site of the deficit. The vessels of the flap are connected microsurgically to the recipient vessels, and the muscle is settled to fill in the defect. In functional free muscle transfer, muscle is harvested with its blood vessels but also with its nerve. Both the vessels and the nerve are connected to the chosen recipients, muscle is reattached to the surroundings to gain the function, and after satisfactory neural and muscular regeneration, the microneurovascular muscle flap begins to function. (Mathes et Hansen, 2006)

In severe facial paralysis treated with functional free muscle transfer, optimal conditions for nerve regeneration via the cross-over nerve graft and the best possible muscle regeneration are crucial to regain mimetic function after paralysis. The functional goals for facial reanimation are to return facial symmetry at rest with oral competence and creation of involuntary smile. Good aesthetic results can be gained with sufficiently small free muscle and with optimal placement of scars. Despite enormous research in the field of surgical technique regarding nerve repair and microneuromuscular flap requirements, there are still a lot of questions to be answered in order to obtain an optimal functional and aesthetic result for the patient.

The present study was designed to elucidate some of the factors affecting the final functional outcome of facial reanimation by two-stage microneurovascular muscle transfer and to characterize the morphology of the nerve grafts and free microneurovascular muscle flaps by radiological and immunohistochemical methods. Better knowledge of clinical and biological parameters functioning in facial reanimation would benefit the patients with facial paralysis.

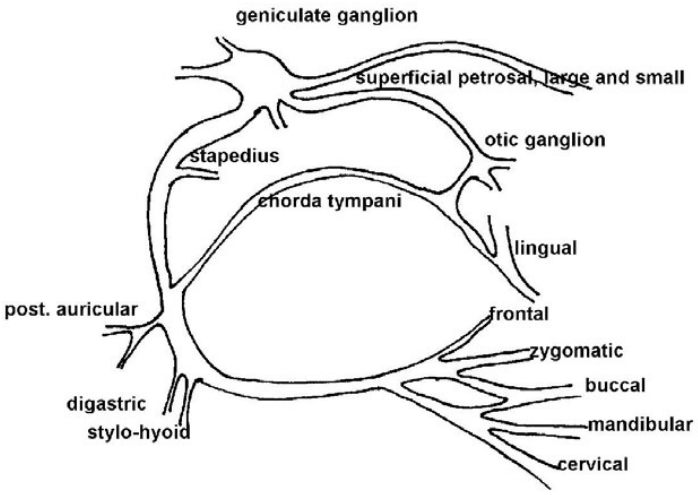
REVIEW OF THE LITERATURE

1. Facial paralysis

1.1 Anatomy

Loss of function of the facial nerve leads to facial paralysis which means paralysis of the muscles innervated by the facial nerve, the seventh cranial nerve. The course of the facial nerve is presented in Figure 1 (modified from Gray's Anatomy, 1977). The facial nerve is the motor nerve of all the muscles of expression in the face, and of platysma and buccinator; the muscles of the external ear; the posterior belly of the digastric, and the stylohyoid. It has the four distinct components including the voluntary motor fibers but also parasympathetic fibers to the lacrimal, submandibular and sublingual glands; sensory innervation to the part of the external ear and its chorda tympani branch contributions to taste at the anterior two-thirds of the tongue. Its tympanic branch supplies the stapedius (Myckatyn et Mackinnon, 2004).

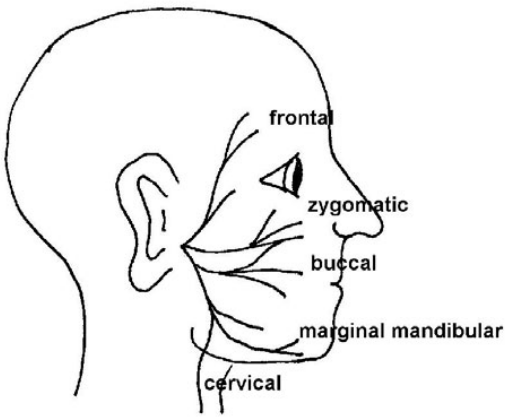
Figure 1. Course of the facial nerve.



For the surgical reconstruction it is necessary to know the course of the extratemporal facial nerve. It leaves the facial canal of the temporal bone coming through the foramen stylomastoideum. It runs in the deep layers below the earlobe, where muscular branches are given off to the occipital, auricular, posterior digastric and stylohyoid muscles. The nerve becomes more superficial before it enters the parotid gland dividing the gland into superficial and deeper parts. Inside the gland, the facial nerve trunk usually divides into superior and inferior divisions, temporo-facial and cervico-facial branches. The facial nerve, running more superficially and distally after the parotid part, divides further into 8-15 branches. Traditionally, five main portions are called frontal (or temporal), zygomatic, buccal, marginal

mandibular and cervical branches but their exact anatomic locations are somewhat variable (Myckatyn et Mackinnon, 2004). (Figure 2, modified from Myckatyn et Mackinnon, 2004).

Figure 2. Main branches of the facial nerve.



Facial musculature comprises 17 paired muscles, and one unpaired sphincter-like muscle, the orbicularis oris. Coordination between these muscles is essential for subtle facial expression. The major muscles affecting the forehead and eyelids are the frontalis, corrugator and orbicularis oculi. There are two groups of muscles controlling the movement of the lips. The lip retractors include the levator labii superioris, levator anguli oris, zygomaticus major and minor for upper lip, and depressor labii inferioris and depressor anguli oris for the lower lip. The antagonists to these lip-retracting muscles is the orbicularis oris, which is responsible for oral continence and some expressive movements of the lips. The mimetic muscles are arranged in four layers so that buccinator, mentalis and levator anguli oris make up the deepest layer. Except for these three muscles, all the other facial muscles receive innervation from facial nerve branches entering deep surfaces. (Zuker et al, 2006)

1.2 Classification and Etiology

It is estimated that idiopathic facial paralysis affects 13-30 people per 100 000 person years worldwide (Rowlands et al, 2002; Kanerva et Pitkäranta, 2006). Peripheral facial nerve paralysis may cause paralysis of all the above-mentioned mimetic muscles. For the patient, this means brow ptosis, inability to close the eyes, and lower lid drooping causing corneal exposure, desiccation and tears running down the cheek. There is smoothness of the cheek, nasolabial furrow becomes loose and the nostrils can not be dilated. The mouth is drawn to the affected side, causing asymmetry of the face and inability to smile, whistle or grin. Food is collected between cheek and gum, causing problems with oral continence.

Facial paralysis can affect both young and old, and may be congenital or acquired. Paralysis can be unilateral or bilateral, and degree of muscle involvement varies from partial to total paralysis.
The duration of the paralysis also varies: most patients regain the function of the muscles spontaneously and approximately 3-4 percent of all the patients with idiopathic paralysis will have long-term facial paralysis (Kanerva et Pitkäranta, 2006).
Most common aetiologies of facial paralysis are shown in Table 1 (modified from Zuker et al, 2006).

1.3. Assessment of mimetic function

Numerous grading systems have been developed to quantify the extent of facial paralysis and the final functional outcome of the patient after the surgical treatment (House, 1983; O´Brien et al, 1990; Murty et al, 1994; Sargent et al, 1998; Frey et al, 1999; Chee et Nedzelski, 2000; Brenner et Neely, 2004). One of the well-established standard methods is House's grading system (House, 1983). The degree of dysfunction is clearly defined on a scale from one to six as presented in Table 2. The evaluation is based on each patient´s interview, and it is therefore subjective. Computer-assisted methods for assessing mimic function are also developed, but they are more expensive and time consuming as there is a need for special hardware and software when using them (Murty et al, 1994; Sargent et al; 1998; Frey et al, 1999).

Table 1. Aetiology of facial paralysis.

A. Extracranial
   1. Traumatic
      - Facial lacerations
      - Blunt forces
      - Mandible fractures
      - Iatrogenic injuries
      - Newborn paralysis
   2. Neoplastic
      - Parotid tumors
      - Tumors of the middle ear or external canal
      - Facial nerve neurinomas
      - Metastatic lesions
   3. Congenital absence of facial musculature

B. Intratemporal
   1. Traumatic
      - Fractures
      - Penetrating injuries
      - Iatrogenic injuries
   2. Neoplastic
      - Cholesteatoma
      - Acoustic neurinomas (Vestibular schwannomas)
      - Hemangiomas
      - Meningeomas
      - Facial neurinomas
      - Metastatic
   3. Infectious
      - Herpes zoster oticus
      - Acute or chronic otitis media
      - Malignant otitis externa
      - Borreliosis
   4. Idiopathic
      - Bell’s palsy
   5. Congenital

C. Intracranial
   1. Neoplastic
   2. Congenital
      - Absence of motor units
   3. Syndromic
      - Moebius syndrome
      - Hemifacial microsomia

4. Iatrogenic injury

Table 2. House scale (House, 1983)

Grade	Definition
I. Normal	Normal facial function in all areas.
II. Mild dysfunction	Slight weakness noticeable only on close inspection. At rest: Normal symmetry and tone. Motion: some to normal movement of forehead; ability to close eye with minimal effort and slight asymmetry; ability to move corners of mouth with minimal effort and slight asymmetry. No synkinesis, contracture or hemifacial spasm.
III. Moderate dysfunction	Obvious but not disfiguring difference between two sides; no functional impairment; noticeable but not severe synkinesis, contracture or hemifacial spasm. At rest: normal symmetry and tone. Motion: slight to no movement of forehead; ability to close eye with maximal effort and obvious asymmetry; ability to move corners of mouth with maximal effort and obvious asymmetry. Patients with obvious but not disfiguring synkinesis, contracture or hemifacial spasm are Grade III regardless of degree of motor activity.
IV. Moderately severe dysfunction	Obvious weakness or disfiguring asymmetry. At rest: normal symmetry and tone. Motion: no movement of forehead; inability to close eye completely with maximal effort; asymmetrical movement of corners of mouth with maximal effort. Patients with synkinesis, mass action or hemifacial spasm severe enough to interfere with function are Grade IV regardless of degree of motor activity.
V. Severe dysfunction	Only barely perceptible motion. At rest: possible asymmetry with droop of corner of mouth and decreased nasal labial fold. Motion: no movement of forehead; incomplete closure of eye and only slight movement of lid with maximal effort; slight movement of corner of mouth. Synkinesis, contracture or hemifacial spasm usually absent.
VI. Total paralysis	Loss of tone; asymmetry; no motion; no synkinesis, contracture or hemifacial spasm.

2. Conservative treatment of facial paralysis

The clinical picture of facial paralysis may vary a lot between the patients. Approximately 96 percent of all the patients with facial palsy will regain spontaneously the function of the facial nerve (Kanerva et Pitkäranta, 2006).
In randomized controlled studies it has been shown that there is no significant benefit from treating idiopathic paralysis (Bell's palsy) with corticosteroids (Salinas, 2008).
Non-surgical approaches may apply temporary protection to the eye. Lid-taping (particularly when sleeping), eye lubrication using clear watery drops, soft contact lenses, and modification of eye-glasses are examples of this treatment. Physiotherapy and speech therapy to establish control of specific muscles or muscle groups are important elements in both pre- and postoperative treatment protocols (Byrne et al, 2004). This consists of various treatment modalities such as biofeedback and self-directed mirror exercises using slow, small and symmetric movements (Zuker et al, 2006).

3. Surgical treatment of facial paralysis

There are several ways of reanimating the face after facial paralysis depending on the aetiology, the duration of the palsy, and the patients' age and life expectancy. Also, the opinion of the patient is important as there are many different options for treating facial paralysis, within certain limits. Timing of the surgery related to the duration of paralysis, condition of the nerve stump and possible tumour involvement within the facial nerve determines the method of surgical treatment. In general, the aims of treatment are to protect the eye, to provide symmetry at rest, and to provide movement. The goals of treatment for the eye are to maintain vision, to provide protection, to maintain function of the eyelids, to improve cosmesis, and to enable the eye to express emotion. The goals for the mouth are to correct asymmetry, to provide oral continence, to improve speech and to provide a balanced symmetric smile (Zuker et al, 2006).
Surgical methods can be divided into static and dynamic procedures. Static procedures include supportive slings (fascia lata or alloplastic sheets), brow or forehead lifts, facelifts, lower lip wedge resections and use of botulinum toxin (Shindo, 1999). Dynamic procedures are presented more detailed in this review.

3.1 Direct nerve repair

Lacerations and iatrogenic injuries of the facial nerve are best repaired immediately in order to achieve the ideal results. The basic principles in peripheral nerve repair apply also to the facial nerve. Microsurgical technique and instrumentation, adequate preparation of nerve ends, avoidance of tension at the suture line and use of smallest calibre sutures that will secure interfascicular and epineurial repair (Millesi, 1972).
It is within the first 30 days after the injury that the neural and muscular elements have the best chance of complete recovery (Conley et May, 2003). In addition, the anatomy is not

hampered by fibrosis and scarring. When repair is delayed beyond that time, the results diminish in strength and quality but are still acceptable until a year (Conley et May, 2003).

3.2 Nerve grafting

The most commonly used donors for nerve grafts are the great auricular, sural, lateral cutaneous femoral nerves or branches of the cervical plexus from the ipsilateral side of the neck (May, 2003a; Lee et al, 2008). Great auricular and sural nerves are ideal to repair the facial nerve in terms of their availability, axon content and length (May, 2003a). In cases where there is a loss of the main trunk or peripheral parts of the facial nerve secondary to trauma, scarring and tumour resection, primary nerve grafting may be the treatment of choice (Tate et Tollefson, 2006). Grafting requires survival of motor end plates in the target muscles which are not present after approximately two years due to motor end plate fibrosis and atrophy (Tate et Tollefson, 2006). The expected rate of axonal regeneration is approximately 1 mm/day, and adequate facial tone can be achieved after 6 months and restoration of motion 1-3 months later (Shindo et al, 1999).

3.3 Cranial nerve substitution techniques

Several procedures have been advocated for late restoration of facial function. When the proximal stump of the facial nerve is not available, the cranial nerve substitution is considered, particularly when repair can be achieved within one to two years after injury (Conley et May, 2003). Although involuntary mimetic movement is not possible with these procedures except when using the contralateral facial nerve (VII cranial nerve), they provide good voluntary facial movement and symmetry at rest. Facial movements develop within 4 to 6 months of the nerve substitution, improving for up to several years (May, 2003b).

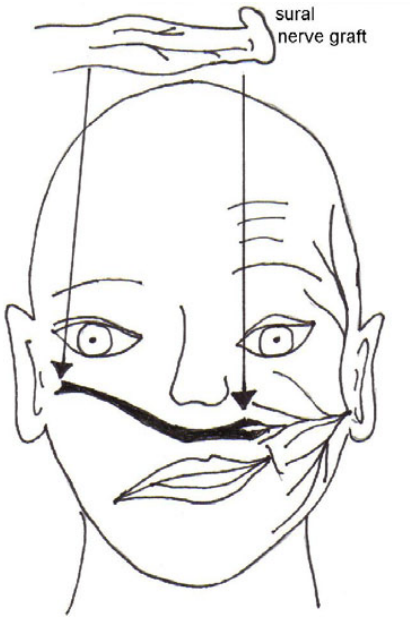
These procedures include spinal accessory nerve cross-over (Drobnick, 1902), cross-facial nerve grafting, (Smith, 1971; Scaramella et Tobias, 1973; Anderl, 1973), ipsilateral hypoglossal nerve cross-over (Conley et Baker, 1979), ipsilateral hypoglossal nerve cross-over with a jump graft (May, 2003b) and a babysitter principle using ipsilateral hypoglossal nerve in conjunction with cross facial nerve graft (Terzis, 1990; Kalantarian et al, 1999). The major disadvantage of donor nerve techniques is that a functional cranial nerve must be sacrificed.

The spinal accessory nerve (XI cranial nerve) is not considered as a first choice for the donor nerves for facial reanimation. Complete sectioning of the nerve can produce significant shoulder weakness, and also unpleasant mass contractions of the facial muscles are possible. (Klebuc et Shenaq, 2004).

The use of the entire hypoglossal nerve (XII cranial nerve) frequently leads to a paralysed, atrophied hemitongue with consequences like impaired talking, chewing, and swallowing. Moreover, hypoglossal–facial transfer can be associated with mass movements of the reinnervated face, setting new demands on central nervous system plasticity (Bernat et al, 2006). The functional deficit produced by complete XII-VII transfer can be reduced by utilizing a jump graft technique. An interposition nerve graft is sutured in an end-to-side fashion to nerve XII, followed by an end-to-end repair to nerve VII (Klebuc et Shenaq, 2004).

Cross-facial nerve grafting procedure was described by several surgeons (Smith, 1971; Scaramella et Tobias, 1973; Anderl, 1973). A nerve graft is placed as a conduit between the branches of the healthy, contralateral side of the facial nerve and corresponding facial nerve branches on the paralysed side as presented in Figure 3 (modified from Zuker et Eppley, 2000). The goal is to provide coordinated spontaneous mimetic function. The disadvantage of this procedure is that it sacrifices normal facial function on the healthy side for the potential benefit of the paralysed side. The more distal the branches used on the normal side, the less efficacy is provided; the more proximal, the greater is the donor effect. The regenerating axons must cross two suture lines and traverse a nerve graft of up to 24 cm in length. A significant size mismatch between the facial nerve branches and nerve graft usually exists. (Klebuc et Shenaq, 2004). Cross-facial nerve grafting also requires a long time, usually 8 to 12 months, for nerve regeneration to reach cross the face to contralateral denervated facial muscles in critical need of trophic support (Tate et Tollefson, 2006).

Figure 3. Cross-facial nerve graft



Cross-facial nerve grafting is particularly best suited for cases of incomplete facial paralysis of less than 6 months duration. It can also be used for individuals with complete hemifacial paralysis of less then 1 year duration. In these cases the cross-facial nerve graft is utilized to reinnervate the orbicularis oculi muscle and for example masseteric nerve is used to reanimate the corner of the mouth. In addition, it can be used for severe cases in combination with microneurovascular muscle transplants. (Klebuc et Shenaq, 2004).

Terzis introduced the “babysitter” procedure, which is a combination of cross-facial nerve grafting and partial hypoglossal nerve transfer with an end-to-side coaptation with the ipsilateral facial nerve (Terzis, 1990). The superiority of this procedure is in providing symmetrical and coordinated facial motion using cross-facial nerve grafting while taking advantage of rapid reinnervation from the ipsilateral hypoglossal nerve to maintain the bulk of the facial muscles without leaving the tongue denervated (Kalantarian et al, 1999).

3.4 Muscle transposition techniques

Regional muscle transfer may be a good solution for patients who are not candidates for extensive surgery (May, 2003c). These techniques involve the transplantation of the temporalis muscle (Gillies 1934; Mc Laughlin 1953; Labbe et Huault, 2000), the masseter muscle or both (Rubin, 1977; Conley et Baker,1979). The traditional Gillies' technique comprised a retrograde transfer of the temporalis muscle, so that the origin of the muscle is detached from the fossa temporalis and turned over the zygomatic arch to extend to the oral commisure. The major disadvantage of this technique is the need for a fascial graft and the placement of the temporalis muscle over the zygomatic arch causing bulging (Labbe et Huault, 2000). In addition, to activate a smile, the patient must clench the teeth. Mc Laughlin suggested an antegrade transfer of the temporalis muscle. This technique was further developed by Labbe, who stopped using a fascial graft and instead transferred the temporalis tendon together with a bony part of the coronoid process under the zygomatic arch, tunneled it through the subcutis of the cheek and attached it to the lip commisure (Labbe et Huault, 2000).

The masseter plastia involves transplanting the whole, or the anterior part of the muscle from its insertions on the mandible and re-inserting it around the mouth (Baker et Conley,1979). Good static control of the mouth can be achieved but the major disadvantages with the masseter plastia are that it creates too horizontal and weak action on the lip and commisure and leaves the angle of the mandibulae looking hollow.

Clinical observations suggest that at least partial spontaneous facial muscle reinnervation takes place from the trigeminal pathway after regional muscle plasties (Labbe et Huault, 2000).

3.5 Protection of the eye

Lagophthalmos and dysfunctional tearing are not only troublesome but can lead to painful corneal exposure and ulcerations. The use of upper eyelid gold weights is a standard of care to be combined with other methods of reanimating the face (Tate et Tollefson, 2006). Paralytic ectropion can be corrected by performing wedge excision of the lateral lower eyelid and lateral canthopexy, sometimes even medial canthopexy is required (Shindo, 1999). The eye can be protected also by contralateral frontal muscle transposition or even with microneurovascular muscle transfer using a part of the muscle or a thin separate muscle as platysma (Terzis, 2004).

3.6 Microneurovascular free muscle transfer

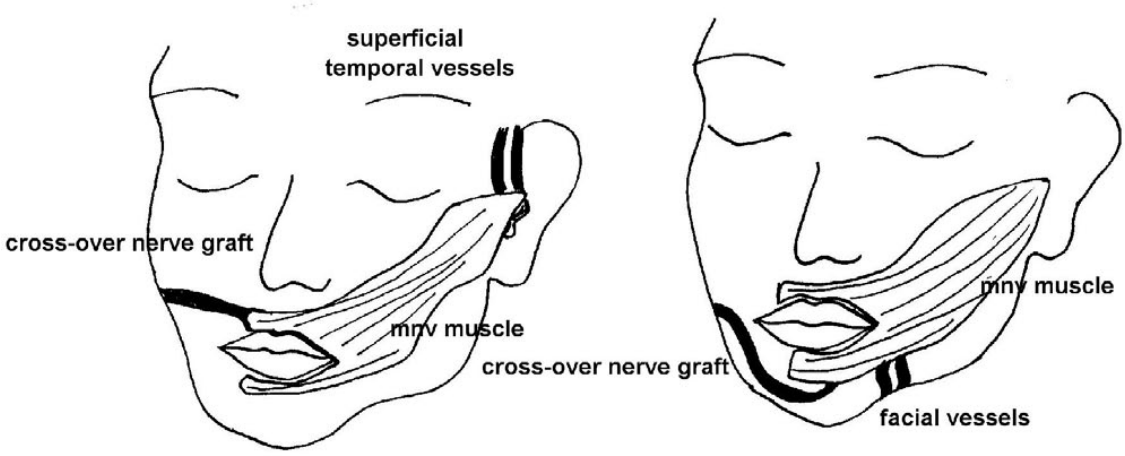
Functioning free muscle transfer is today becoming more and more accepted as the first choice of reconstruction for long-lasting total facial paralysis to restore emotional expression of the face and possibly subconscious smile (Harii et al, 1976; Harrison ,1985 ; O'Brien et al, 1990; Terzis et Noah, 1997; Frey et Giovanoli, 2002; Chuang, 2002). Free muscle transfer is indicated when native facial muscles cannot be reinnervated due to the paralysis longer than two years causing severe atrophy of the mimetic muscles. Younger patients who desire

mimetic dynamic reconstruction and are willing to accept long waiting for the results are optimal candidates for this technique (Swartz, 2003).

The two-stage method to reanimate long-standing facial paralysis by cross-facial nerve grafting and later free microneurovascular muscle transfer has been the standard treatment of choice for almost 30 years (Harii, 1979). Schematic drawing of the procedure proposed by Harii (Harii, 1979) is presented in Figure 4A, and the other possibility to place the grafts is presented in Figure 4B (O'Brien et al, 1980).

Figure 4A and B. Schematic drawings of the possibilities to place the nerve grafts and free muscle transplant in facial reanimation.

Figure 4A. **Figure 4B**



In the first stage the aim is to use the healthy side of the face as a motor donor and by cross-facial nerve grafting bring the innervation to the paralyzed side from the healthy facial nerve. The only technique currently available to restore both voluntary and involuntary facial movements plus symmetry at rest requires the use of the facial nerve (VII cranial nerve) (Zuker et al, 2006). Preoperative analysis of the type of smiling the patient has on the nonparalysed side is crucial for a symmetrical result. Individuals have various types of smiles, and it is important to assess the direction of movement of the upper lip and commisure (Paletz et al,1993).

The nerve graft, which is usually a sural nerve, is harvested and tunnelled across the upper lip or under the chin to connect the healthy side to the preauricular area on the paralyzed side. It may be coapted to contralateral facial nerve either end-to-end or end-to-side (Terzis et Noah, 2002; Frey et al, 2006).

Many free muscle flaps have been used for facial reanimation: gracilis (Harii, 1976; Terzis et Noah, 1997; Frey et Giovanoli 2002), pectoralis minor (Harrison, 1985; Terzis, 1989), latissimus dorsi (Harii, 1979), serratus anterior (Whitney et al, 1990), rectus femoris (Koshima et al, 1994), rectus abdominis (Hata et al, 1990), extensor digitorum brevis of the foot (Mayou et al, 1981), abductor hallucis (Jiang et al, 1995) and external oblique muscle (Kuzbari et al, 1997). The most commonly transferred muscles are the gracilis, latissimus dorsi and pectoralis minor, the latter is used with good results especially in paediatric patients (Terzis et Noah,1997; Harrison, 2005).

In the second stage, that usually is performed 8-12 months after the first operation, when a positive Tinell sign can be recalled at the distal end of the cross-facial nerve graft, a microneurovascular muscle transplantation is performed. The carefully tailored muscle graft is brought to the cheek area with the vascular and neural pedicles superficially. It is fastened with its distal parts fan-shaped divided into the upper and lower lips, sometimes with a small part placed into the alar base. The proximal part can be pulled gently to ascertain a smile. The upper fibers are brought as close to the zygomaticus as possible and inserted into the malar area (Harrison, 2002). The muscle is then spread out along the zygomatic arch into the SMAS in the preauricular region and down to the sternomastoid fascia to the lowest part. (Harrison, 2002). Vascular anastomoses are performed to the facial or the superficial temporal vessels, depending on which muscle is used and how it is placed. Nerve anastomosis is performed to the clean cut distal end of the cross-facial nerve graft (Chuang, 2002).

Above is described one possibility of performing a two-stage procedure in facial reanimation. The technical details may vary according to the patient and the surgeon.

The patient is monitored for circulation problems in the flap, postoperative haematoma or infection. When the postoperative period is uneventful, function appears in the muscle graft 6-12 months after transfer providing satisfactory elevation of the nasolabial fold corresponding well with contralateral facial movements (Harii et al, 1979).

Some authors recommend electric stimulation of the muscle graft until the muscle begins to contract. The active muscle training should be started when the muscle can be controlled by the patient, often with the help of physiotherapist. Active muscle training should be continued to gain not only muscle contraction but also coordinated facial movements. (Frey et Giovanoli, 2002)

Results after the two-stage reanimation are usually from moderate to good varying in different patient series as many factors contribute to the functional outcome (O´Brien et al, 1990; Sassoon et al, 1991; Terzis et Noah, 1997; Kumar et Hassan, 2002). Restoration of the original resting tension of the muscle graft (Terzis et al, 1978; Frey et al, 1983) and meticulous coaptation of the nerve (Manktelow et al, 1984) between the donor nerve and the motor nerve of the muscle graft have proved to be of great importance. Failures can be attributed to vascular thrombosis, lack of innervation or both. Morover, optimal results depend on muscle inset to the face, bulkiness and strength of the muscle (Swartz, 2003).

Possible late problems with microneurovascular muscle transfer include persistent bulkiness of the muscle transplant and synkinesis or mass movements in the face. Donor-side sequelae after harvesting the sural nerve may result in numbness of the lateral area of the foot, pain or scar hypertrophy (Zuker et al, 2006). Synkinesis is defined as the abnormal, simultaneous contraction of a group of muscles with voluntary or involuntary facial expression. This phenomenon occurs when regenerating axons innervate unintended targets (Rovak et al, 2004a).

Nowadays, microneurovascular reanimation of the face can be carried out also in one stage (Chuang, 2002). In the one-stage procedure, microneurovascular muscle with a longer neural pedicle such as latissimus dorsi or rectus abdominis is connected to the healthy side of the facial nerve directly without the cross-over nerve graft (Harii et al, 1998; Kumar et Hassan,

2002). The advantages are that the patient undergoes only one operation , no nerve graft is needed, and mimetic function reappears sooner (Takushima et al, 2002). The disadvantages of the one-stage procedure are the limited freedom of positioning the muscle graft and possible problematic scar development (Guelinckx et Sinsel, 1996; Rab et al, 2006).

When there is neither an ipsilateral nor a contralateral facial nerve available to act as a donor, as for example in the classical Moebius patient, other motor donors must be used to innervate the microneurovascular muscle flap. Objective testing of bilateral cranial nerve motor function and electrophysiological tests are crucial in order to avoid causing further impairment to the patient by reconstructive surgery. The facial nerve excluded, possible motor donors are the trigeminal (V), the spinal accessory (XI), and the hypoglossal (XII) nerves. The hypoglossal nerve is often affected in Moebius patients, and should therefore be left intact (Terzis et Noah, 2002). The masseter branch of the trigeminal nerve is often used while gracilis transfer to this nerve can be performed in one stage (Zuker et al, 2000; Terzis et Noah, 2002) In the majority of children and in 50 % of adult patients, due to possible reorganization of the cerebral cortex, this procedure can provide a symmetric smile with exellent muscle excursion without conscious effort or biting motion (Zuker et al, 2000). In rare cases of Moebius syndrome the cervical or brachial plexus can be used as motor sources (Terzis et Noah, 2002).

In most cases, after microneurovascular reconstruction, a tertiary refinement procedure will be needed later. Depending on the patient and the functional result, these procedures include brow-lifts, face-lifts, blepharoplasties, repositioning of the grafted muscle, static slings or functional coupling by botulinum toxin (Zuker et Eppley, 2000; Tate et Tollefson, 2006).

4. Structure of free muscle flaps in magnetic resonance imaging

Magnetic resonance imaging (MRI) is an accurate imaging method for evaluating muscles (Murphy et al, 1986; Lamminen et al, 1990; Fleckenstein et al, 1993). Clinical MRI studies oo normal skeletal muscles have obtained clear delineation between muscle tissue, intermuscular fat, subcutaneous fat and cortical bone in T1-weighted, proton density-weighted and T2-weighted images (Murphy et al, 1986; Fisher et al, 1986) . Subcutaneous fat has been shown to be a useful and stable reference tissue for signal intensity evaluations in MRI (Dooms et al, 1986). MRI studies in patients with different neuromuscular diseases show different patterns of changes in the size and signal intensity of muscles (Murphy et al, 1986; Lamminen et al, 1990; Hudgins et al, 1994).

Many studies have been conducted on the MRI appearance of denervated skeletal muscles including both traumatic and postoperative neuropathies and denervated free microvascular muscle flaps (Murphy et al, 1986; Lamminen et al, 1990; Fleckenstein et al, 1993; Salmi et al, 1995; Chong et al, 2001; Fischbein et al, 2001; Bendszus et al, 2002).

The changes that accompany motor denervation include asymmetrical decrease in affected muscle volume, fatty infiltration and variable signal intensity changes as T1 and T2 prolongation and postcontrast enhancement (Fleckenstein et al, 1993; Uetani et al, 1993; Russo et al, 1997).

In clinical denervation studies paralyzed muscles showed high signal intensity on T2-weighted images and showed marked atrophy and fatty infiltration on T1-weighted images (Fleckenstein et al, 1993; Uetani et al, 1993). In some patients, MRI findings became normal after recovery of the paralysis and reinnervation (Fleckenstein et al, 1993; Uetani et al, 1993). In a study describing MRI findings in two patients with perineural spread of tumours, T2 prolongation was seen in facial muscles, suggesting denervation changes (Fischbein et al, 2001).

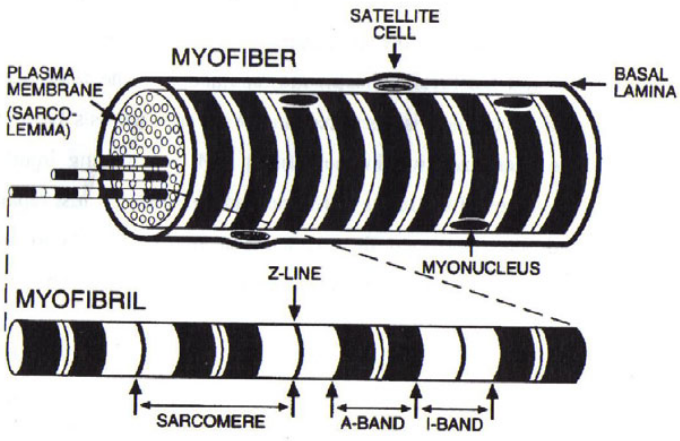
Denervated microvascular free flaps have been studied with MRI (Hudgins et al, 1994; Salmi et al, 1995; Wester et al, 1995; Chong et al, 2001). Nerve supply to the microvascular flap is surgically interrupted, leading to muscle atrophy, which results in a predominantly fatty appearance of the flap on MR images (Wester et al, 1995; Salmi et al, 1995). Immediately after the operation, the microvascular flaps demonstrated mostly intramuscular oedema and the volume of the flaps increased by 2 weeks postoperatively. At 6 months, the flaps had returned to their initial volumes with evident fat degeneration (Salmi et al, 1995). In a study by Chong with 20 free flaps and seven pedicled flaps, there was no statistically significant difference between pedicled and free flaps in terms of enhancement, T2 signal or tendency to appear striated. Despite denervation, most of the flaps in this series did not atrophy over three years follow-up time (Chong et al, 2001).

5. Morphology of skeletal muscle tissue

Skeletal muscles generate force and movement, and are under voluntary control. Skeletal muscle is composed of specialized cells called myofibers. They are separated by a sheath termed endomysium. In turn, myofibers are arranged in fascicles which are surrounded by a connective tissue layer called perimysium. The whole muscle is covered in a third connective tissue sheath, epimysium. Myofiber is found to be composed of parallel myofibrils. The myofibrils lie within the sarcoplasm which also contains cellular organelles such as mitochondria, endoplasmic retinaculum, t-tubules, ribosomes and glycogen particles (Figure 5). The basic contractile unit within each myofibril is called a sarcomere, consisiting of thin and thick filaments that are made of actin and myosin proteins, respectively, and other myofibrillar proteins accounting for the striated appearance of skeletal muscle. Each motor neuron innervates a group of myofibers which together form a motor unit. Thus, each fiber within a motor unit is innervated by a single alpha motor neuron which terminates in a neuromuscular junction, also called a motor end plate. The myofibers of each motor unit contract simultaneously (Rudkin et Miller, 2006).

Skeletal muscle in mammals consists of different types of fibers. The functional demand of a muscle determines the fiber types and fiber type distribution in a muscle. There are slow- (type 1) and fast- (type2) twitch types of fibers. Type 2 fibers are further subdivided into groups type 2A (fast oxidative) and types 2B and 2X (fast glycolytic). In humans, most muscles contain a mixture of fiber types, and one fiber type can predominate in some muscles.(Rudkin et Miller, 2006; Äärimaa, 2006).

Figure 5. Structure of myofiber (reproduced with the permission of M. Kääriäinen, 2001)



5.1. Denervation and reinnervation changes in skeletal muscle tissue

Alterations in neuronal input, vascular supply, resting length and muscle activity will produce alterations in muscle morphology (Guelinckx et al, 1997).

Experimental studies have shown that both muscle transfer and denervation cause trauma to muscle tissue. It is established that the morphology, physiology and biomechanical

characteristics of muscle fibers are determined by the type of innervating motor neuron (Cote et Faulkner, 1986).

The trophic status of the muscle is directly related to the integrity of the motor nerve, so it is obvious that nerve injury will produce changes in the physiological, morphological and histochemical characteristics of the affected muscle.

The denervation of muscle results in muscle atrophy, as manifested by a reduction in muscle fiber size and number, and at the same time an increase in connective tissue (Zhang et al, 1997). The mass of the denervated muscles usually decreases to approximately 30 percent of the initial mass. The motor nerve is able to arrest the atrophic changes of denervated muscle and preserve the muscle mass to about 90 percent of its initial weight (Zhang et al, 1997). The principal morphological change of denervated muscle fibers is shrinkage of the cross-sectional area of the fibers. (Carlson et Faulkner, 1983; Carlson et Faulkner, 1988; Carlson et Faulkner, 1989; Schmalbruch et al, 1991; 1994). Also fiber type grouping and changing the relative number of the fiber types after the denervation and reinnervation are well known characteristics (Guelinckx et al, 1997; Jakubiec-Puka et al, 1992). Microscopic changes in denervated myofibers closely resemble those in immobilized muscles (Cooper, 1972) as disuse alone can cause atrophy, as seen with extremity cast immobilization and tendon transsection (Kauhanen et al, 1996).

Skeletal muscle is capable of extensive regeneration after injury. Satellite cells, located between the basement membrane and plasma membrane of the myofiber, are responsible for the regeneration process (Grounds, 1991). They are quiescent myogenic precursor cells, which become activated following muscle injury. Satellite cells proliferate and differentiate into myoblasts to fuse to myotubes and finally mature into adult muscle fibers (Bodine-Fowler, 1994) Even without nerve supply, satellite cells can divide and form myotubes (Schmalbruch et al, 1991). Reinnervation, however, is necessary for the new fibers to mature (Schmalbruch et Lewis, 1994; Rantanen et al, 1995). In muscle lacking contractile stimuli or complete innervation, repeated attempts at regeneration will eventually cause exhaustion of the myogenic precursor cells, i.e. satellite cells (Campion, 1984; Schmalbruch et Lewis, 1994; Rantanen et al, 1995; Jejurikar et al, 2002). Satellite cell depletion, in turn, causes impairment of contractile function and loss of the ability to repair muscle fibers (Schmalbruch et Lewis, 1994; Jejurikar et al, 2002).

A key determinant of the success of both the regeneration and the subsequent innervation of muscles is the basal lamina. The basal lamina sheaths surrounding each muscle and nerve fiber provide a mechanical scaffold and supporting matrix that promotes interactions between muscle and nerve cells during regeneration. In addition to its general supporting role during regeneration, the basal lamina acts as substrate for the attachment of important signaling molecules at the neuromuscular junction. The most important of these is agrin. Agrin survives the regeneration of both nerve and muscle cells, and serves to promote and coordinate effective reconstruction of neuromuscular junctions on the regenerated muscle fibers (Slater et Schiaffino, 2008).

5.2 Morphology of free microvascular muscle flaps

The issue of morphological changes in free flaps is more complicated than in denervated muscle as, with the flaps, there is also the disturbed vascularity, decreased muscle tension and disuse of the muscle flap. In general, it is believed that morphological changes in non-reinnervated flaps are similar to those seen in denervated muscle tissue (Zhang et al 1997).

In a human study, mean muscle fiber diameter decreased significantly and muscle type 1 atrophy was dominant, increasing the percentage of type 2 fibers after the muscle transfer. Fatty change and fibrosis were also present and they increased with the duration of follow-up. (Kauhanen et al, 1998a; Kauhanen et al, 2004) Predominant type 1 atrophy is reported also in experimental models (Roy et al, 1992).

Reinnervation can occur spontaneously from nerves in surrounding tissues, a phenomenon called neurotization, in microvascular free flaps (Kauhanen et al, 2004). In human material, satellite cells were found even 3-4 years after free flap transfer, indicating continuous regeneration of the muscle (Kauhanen et al, 2004).

5.3 Morphology of free microneurovascular muscle flaps

In a large experimental study, the denervated, non-transplanted muscles which received both motor nerve reinnervation and neurotization resulted in significantly preserved muscle mass compared with the denervated control muscles. In transplanted gracilis free flaps, however, only direct reinnervation with motor or sensory nerves resulted in improved bulk preservation. Neither sensory nor motor neurotization was significantly effective in the free flap model (Zhang et al, 1997).

In an experimental study on reinnervated prefabricated free flaps, a considerable amount of muscle atrophy was present regardless of the extent of reinnervation (Kostakoglu et al, 1995).

In experimental data, it has been shown that fiber necrosis, degeneration and regeneration do not occur to a significant extent after microneurovascular transfer (Guelinckx et al, 1988; Yoshimura et al, 2006).

In a study by Frey with seven facial reanimation patients, the morphology of microneurovascular muscle grafts correlated well with the functional result. Muscle fiber type distribution showed a strong influence of the quality of the nerve used for the crossover innervation. Morphology of the muscle flaps showed variation: in some biopsies the muscle fibers were regular and scar tissue was scarce wheras in others atrophic fibers were predominant (Frey et al, 1991).

Yoshimura et al. showed in a series of nine patients that there were regularly distributed fibers and fiber type grouping indicating successful reinnervation after microneurovascular muscle transfer. Satellite cells were responsive even ten years after the microneurovascular muscle transfer, suggesting that muscle regeneration might still take place long after transfer even if the muscle fiber area and diameter were similar to that of control muscles (Yoshimura et al, 1998).

6. Degeneration and regeneration of the peripheral nerve

Peripheral nerve is composed of somesthetic and sympathetic sensory and motor fibers. The nerves themselves are composed of axons which can be either myelinated or unmyelinated, and are supported by Schwann cells. Endoneurium surrounds individual axons and their Schwann cell sheaths and is predominantly composed of orderly oriented collagen fibers. Next, the perineurium, formed from many layers of flattened cells (e.g. fibroblasts) and collagen, surrounds groups of axons to form fascicles. Finally, epineurium, an outer sheath of loose fibrocollagenous tissue, binds individual nerve fascicles into a nerve trunk. Peripheral nerves are well vascularized by vessels in the surrounding tissue, penetrating the nerve and continuing as capillaries within the supporting tissue of the nerve trunk. (Schmidt et Leach, 2003; Shenaq et Kim, 2006).

In general, facial nerve behaves like any peripheral motor nerve in its capacity to regenerate (Choi et Dunn, 2001). After nerve injury the, Wallerian degeneration takes place so that the distal axon and myelin begins to degenerate, and finally debris is phagocytosed by macrophages and Schwann cells (Choi et Dunn, 2001; Schmidt et Leach, 2003). In addition to clearing axonal and myelin debris, Schwann cells produce cytokines and many neurotrophic factors and their receptors, including NGF and p75NGFR, thus enhancing axonal growth (Frostick et al, 1998; Choi et Dunn, 2001; Schmidt et Leach, 2003). After debris clearance, regeneration begins from the proximal surviving part of the severed nerve to the distal stump. Schwann cells ensheath regenerating axons and later myelinate them (Choi et Dunn, 2001).

6.1 Histological findings in nerve grafts

Nerve grafting or a nerve conduit between the ends of the transected nerve is required when the gap between the stumps of the nerve is so long that suturing them together would lead to harmful tension and thus impair regeneration (Millesi et al, 1972; Terzis et al, 1997). In humans, usually in nerve gaps more than 2.0 cm to 2.5 cm, nerve grafting needs to be considered (Millesi, 1987). Axons in these autogenous nerve grafts will undergo Wallerian degeneration but retain the critical connective tissue scaffold for ingrowing axon sprouts (Millesi, 1991).

There has been a lot of experimental work studying the effects of other materials serving as conduits than autologous nerve grafts, but so far none of the materials studied have either matched or exceeded the performance of natural nerve grafts (Schmidt et Leach, 2003).

Some experimental work has aimed to characterize the morphology of free nerve grafts and attempted to clarify the factors which influence the final function of microneurovascular muscle flaps (Frey et al, 1992; Koller et al, 1993; Frey et al, 1998). In a study in sheep, the mean diameter of axons in nerve grafts was about one-quarter of the mean diameter of axons in the normal motor nerve. No clear correlation was found between the time from nerve grafting to biopsy and the number of axons in the distal end of the nerve graft (Frey et al, 1992). However, there was an up to fourfold increase in the number of axons at the distal end of the graft (Frey et al, 1992). In a rat model, cross-facial nerve grafts were examined histomorphometrically, revealing decreased axon diameter and mean myelin area in the nerve

grafts compared to control nerves (Thanos et Terzis, 1996). In this experimental study, there was a positive correlation between number of axons in the graft and motor endplate counts in reinnervated muscle. (Thanos et Terzis, 1996).
Previously, small numbers of human cross-facial nerve grafts have been evaluated histologically showing increased fibrosis of the grafted nerve (Vedung et Olsson, 1982; Frey et al, 1991; Frey et al, 1996). In a clinical study of five patients, a large number of unmyelinated axons was seen in the distal end of sural nerve grafts, and the myelinization increased as the time between nerve grafting and biopsy taking was up to one year (Vedung et Olsson, 1982). In another series with seven patients, number of regenerated nerve fibers was only 20 % of the original number of nerve fibers, and they were consistently thinner (Frey et al, 1991; Frey et al, 1996). In a quantitative study on sural cross-over nerve grafts, myelinated fibers remained small and the numbers of regenerating axons did not correlate with age nor regeneration time (Jacobs et al, 1996).

6.2 Expression of P75NGFR in neural tissue

Nerve growth factor (NGF) was the first neurotrophic factor, isolated in 1951 from male mouse salivary glands (Levi-Montalcini et Hamburger, 1951), but much later it was believed to be of great importance in the adult nervous system (Hefti, 1986). NGF is expressed at low levels in healthy peripheral nerve by Schwann cells and is upregulated in the distal stump upon injury (Saika et al, 1991). NGF promotes the survival and outgrowth in sensory neurons, but its role in motor nerve regeneration is unsure (Tuszynski et al, 1996; Oudega et al, 1996).
So far, two distinct receptors have been identified for NGF: a low-affinity nerve growth factor receptor (p75NGFR) and a high-affinity receptor (trkA) (Frostick et al, 1998; Choi et Dunn, 2001).
The trk family of receptor tyrosine kinases, (trkA, trkB and trkC) includes high-affinity receptors with a high specificity for individual neurotrophins (Klein et al, 1991; Squinto et al, 1991).
P75NGFR binds to all neurotrophins and interacts with all receptors of the trk family (Dechant et al, 1997). P75NGFR is expressed during early neuronal development, and is thought to be important for motoneuronal survival during development (Boyd et Gordon, 2001; Dechant et Barde, 2002).
In the adult, p75NGFR is re-expressed in various pathological conditions including mechanical trauma, focal ischaemia, stroke, epileptic seizures and Alzheimer's disease (Dechant et Barde, 2002). In experimental work, p75NGFR is markedly increased in axotomized motoneurons (Ernfors et al, 1989; Rende et al, 1995; Gschwendtner et al, 2003) or after spinal root avulsion (Wu et al, 2004). In a study in rat, the expression of nerve growth factor receptor was greatly increased after the sciatic nerve crush decreasing to control levels by the time the motor function of the leg had been restored (Ernfors et al, 1989). In a more recent study, the expression of p75NGFR was examined during peripheral transsection of the facial nerve of adult mice. Axotomy led to a strong increase in p75NGFR immunoreactivity on the injured and regenerating facial motoneurons and on denervated Schwann cells (Gschwendtner et al, 2003).

However, function of this receptor in normal adult motoneurons remains unknown (Ferri et al, 1998; Dechant et Barde, 2002). It has been suggested that p75NGFR participates in apoptosis of motoneurons and Schwann cells (Ferri et al, 1998; Ferri et Bisby, 1999; Dechant et Barde, 2002; Boyd et Gordon, 2003;) but on the other hand, it has a role in the survival of motoneurons by interacting with specific receptors (Ferri et al, 2002).
In a study using nerve grafting after spinal root avulsion in adult rats, all regenerating motoneurons expressed p75NGFR while all motoneurons that failed to regenerate did not express p75NGFR. (Wu et al, 2004). In another study on sciatic nerve transsection in rats, the Schwann cells in the distal segment showed a prolonged period of NGF and p75NGFR expression (Liu et al, 1995).

6.3.Expression of VEGF in neural tissue

Vascular endothelial growth factor, VEGF, has a predominant role in the formation of vascular structures (angiogenesis) during embryonic development and in numerous pathological conditions (Carmeliet et Storkebaum, 2002; Storkebaum et al, 2004). It was discovered over 20 years ago (Senger et al, 1983) as a vascular permeability factor, and was later cloned and termed vascular endothelial factor by Ferrara et al. (Ferrara et Henzel, 1989). VEGF binds the receptor tyrosine kinases Flt-1 (VEGFR-1) and Flk-1 (VEGFR-2/KDR). Neuropilin-1(NP-1) is a specific receptor to a certain VEGF isoform (VEGF 165) and a co-receptor of Flk-1 (Soker et al, 1998). More recent findings reveal that VEGF has direct effects on neural cells (Sondell et al, 1999a; Hobson et al, 2000; Carmeliet et Storkebaum, 2002; Storkebaum et al, 2004).
Hobson showed that neovascularization preceded axonal regeneration in rats in an acellular nerve conduit composed of orientated fibronectin mat. In that model, nervous tissue was absent from avascularized areas whereas Schwann cells and axons extended together and were most numerous in well vascularized areas (Hobson et al, 1997). Regenerated nerve fibers were found more frequently in the vicinity of the blood vessels also in an earlier study (Weis et Schröder, 1989).
Experimental work, both in vitro and in vivo, has shown that VEGF and its receptors exist in neural cells (Sondell et al,1999a; Sondell et al,1999b; Oosthuyse et al, 2001; Islamov et al, 2004). In adult mouse spinal cord, VEGF was produced by motor neuron itself or nearby astrocytes, and Flk-1 and NP-1 were co-expressed in motor neurons (Oosthuyse et al, 2001). Sondell and coworkers demonstrated expression of VEGF in cultured mouse nervous tissue, and also expression of Flk-1 receptor in dorsal root ganglia and Schwann cells (Sondell et al, 1999a). In an experimental model, mice were submitted to experimental nerve crush injury, and the expression of VEGF and Flt-1 was increased compared to controls. However, VEGF, Flk-1 and Flt-1 were also expressed in normal mouse sciatic nerve although in lesser amount (Islamov et al, 2004).
The neurotrophic effect of VEGF would appear to be mediated mostly through the receptor Flk-1 (Zhang et al, 2003). In abundant studies, VEGF has been added to neural structures to elucidate its effect on nerve regeneration (Sondell et al, 1999a; Sondell et al, 1999b; Hobson et al, 2000; Rovak et al, 2004b). Added VEGF increased the number of Schwann cells and

enhanced axonal regeneration in cultured mouse dorsal root ganglia (Sondell et al, 1999a). In one study, VEGF was added to acellular nerve conduits with variable results. The total number of axons was increased at the proximal nerve gap coaptation site, but there were relatively few axons at the distal coaptation site (Rovak et al, 2004b). In another study, VEGF stimulated the outgrowth of blood vessels and Schwann cells but not axons in acellular nerve conduit (Sondell et al, 1999b), and in yet another study, addition of VEGF to a silicone nerve chamber resulted first in increased blood vessel formation and later in increased axonal regeneration and Schwann cell migration (Hobson et al, 2000).

Samples are taken rarely from human neural tissue or nerve grafts, so the literature concerning the expression of VEGF in neural tissue is mostly based on experimental work. However, in one clinical study, improvement in chronic ischemic neuropathy after intramuscular VEGF gene therapy in patients with critical lower limb ischaemia was reported (Simovic et al, 2001).

AIMS OF THE STUDY

In this thesis, cross-over nerve grafts and microneurovascular muscle flaps in humans are examined in the clinical context of surgical treatment after severe facial paralysis.

The specific aims of the present study were:

1. To report the long-term functional outcome of facial reanimation using two-stage procedure and to find out which factors influenced the final result.

2. To describe the maintenance of muscular structure and volume of microneurovascular flaps after facial reanimation by MRI.

3. To characterize regenerative structural changes in microneurovascular muscle transplants after facial reanimation using immunohistochemical methods.

4. To document the changes in nerve morphology in a large material of human cross-facial nerve grafts before free muscle transfer.

5. To characterize the expression of specific growth factors and their receptors in human cross-facial nerve graft.

6. To compare the morphological findings of muscle and nerve with the function of the transplanted microneurovascular muscle flap.

MATERIAL AND METHODS

1. Patients (Studies I-V)

This thesis comprise a total of 37 patients who presented with complete, long-lasting unilateral facial paralysis between 1986 and 2004 in Helsinki University Hospital. Twenty-seven patients (study I) responded to an invitation to attend a clinical evaluation including an interview and a video recording. Fifteen patients of the 27 were able to participate in MRI study (study II). The etiology of the paralysis of 37 patients (study IV) is presented in Table 3. Etiology of the specific subgroups of patients in studies I to IV is presented in original articles. The duration of the facial paralysis preoperatively ranged from 1 to 41 years. The age of patients varied from 7 to 65 years at the time of operation. Only one patient was a child, a girl aged 7 years.

Table 3. Etiology of facial paralysis of 37 patients (Study IV)

Removal of acoustic neurinoma	16
Parotidectomy	5
Trauma	3
Intra-acoustic diseases	3
Removal of intracranial tumors	2
Congenital	4
Not known	4

Total	37

Seventeen (Study III), 37 (Study IV) and 35 (Study V) patients participated in immunohistochemical studies.

All patients were operated on by a senior surgeon using a standard two-stage method. In the first stage, an 18-20 cm long sural nerve graft was harvested and transplanted under the chin to connect the healthy side to the preauricular area on the paralysed side. Large buccal branches of the facial nerve (8-12 axons) on the healthy side were sacrificed, and the nerve graft was coapted epineurially with 10/0 sutures. Eight months (range 4-20mos) after the nerve transplantation, the microneurovascular muscle bundle was grafted. The size, length, and total volume of the graft were carefully planned. Blood vessels were anastomosed to the facial artery and vein under the chin. The nerve of the muscle was coapted to the nerve graft with epineurial sutures. From 6 to 8 months later, patients started to train the muscle transplant. They were advised to exercise their mimic muscles in front of the mirror several times a day.

The sural nerve was used as a cross-facial nerve graft in every case. Three different muscles: gracilis, latissimus dorsi, serratus anterior were used as a microneurovascular muscle flap.

To evaluate which demographic and clinical factors influenced the final functional outcome of facial reanimation the patients' hospital charts were reviewed retrospectively. The data of characteristics of all the patients and their operations in individual studies (I-V) is presented in Table 4.

After transplantation of the muscle, other procedures including tarsorraphies (n=16) and forehead procedures (n=4) were needed 53 times in 21 patients (study I). Secondary operations involving the transplanted muscle totalled 32 facial lifts including correcting the position of the free muscle in 19 patients during follow-up (study I).

Table 4. Characteristics of patients and operations

	Study I	Study II	Study III	Study IV	Study V
Number of patients	27	15	17	37	35
Age at the time of mnv muscle transfer	7-65y mean 41y	7-63y mean 36y	7-62y mean 41y	7-65y mean 38y	7-65y mean 38y
Time between nerve cross-over and mnv muscle transfer	4-20m mean 8m	4-19m mean 8m	4-20m mean 7m	4-20m mean 8m	4-20m mean 8m
Number of patients available for evaluation of functional outcome	27	15	14	24	24
Follow-up time	2-15y mean 8,5y	3-14y mean 7y	2-15y mean 8	2-15y mean 8,5y	2-15y mean 8,5y
Time between muscle transfer and muscle biopsy			1-10y mean 2,7y	1-10y mean 2,7y	1-10y mean 2,7y

2. Evaluation of functional outcome (Study I)

Twenty-seven patients were videorecorded at rest, while speaking, and while making a number of voluntary movements to show mimic muscle function. The functional outcome of the operation was illustrated by the amount of facial dysfunction remaining. It was graded objectively during the interview by a single physician on a scale from 1 to 6 as described by House, Table 2 (House, 1983). These grades were later combined with those obtained from the videotapes. The House scale was modified by disregarding the function of the forehead, as in these operations no muscle was transferred to the forehead area.

In interviews, satisfaction of the patients was measured by asking them how the operations had affected their quality of life. The answers were graded on a scale from 1 to 4:

1) quality of life considerably better after the operations
2) quality of life better after the operations
3) quality of life unchanged after the operation
4) quality of life poorer than before the operations

They were also asked to estimate how much time they spent practicing facial movements after they had been asked to train their facial function. The amount of practice was graded on a scale from 1 (none) to 4 (several times a day).

3. Magnetic resonance imaging (Study II)

Fifteen patients underwent MRI with a 1.5 T unit (Sigma LX, General Electric Medical Systems, Milwaukee, WI) using a standard head coil. The sequences used in this study were axial T2-weighted fast spin echo, coronal and oblique coronal T1-weighted spin echo, and oblique coronal T2 weighted fast spin echo with fat saturation. Axial and coronal images, the oblique coronal images and coronal T1-weighted spin echo images (TR 440 msec/ TE 21 msec, matrix 256x 224, field-of view 18 cm, slice thickness 3 mm) were used to align with the expected course of the free flap, i.e. from the junction of the zygomatic bone and arch to the corner of the mouth. Owing to the small cross-sectional area of the flaps, images perpendicular to the long axis of the flap were not useful in the analysis. Images paralleling the flaps were therefore used in the analysis.

The MR images were viewed on a PACS workstation and evaluated by two investigators blinded to the clinical results. The free flap area of each slice was defined manually (region-of-interest method) from the oblique coronal T1-weighted images, and the area was calculated using the software (AGFA Impax 4.1) provided with the workstation. The volume of the free flap was then calculated from the formula

Volume = (Area 1 x thickness of a MRI slice 1) + (area 2 x thickness of a slice 2)+…+ (area n x thickness of a slice n), where n is the last slice to be calculated.

The maximum thickness of the muscle was also marked. The muscle structure of the free flaps was semi-quantitatively assessed on a scale from 1 to 4 by judging the appearance of the muscle. Grades were from normal or almost normal muscle structure (grade I) to fatty-infiltration (grade II) or severe muscle atrophy and fibrosis (grade III). Grade IV was recorded in one case in which no muscle at all could be identified.

The initial weight of the flap (n=10) ranged from 20 to 75g. The original volume of these free flaps was calculated from the formula:

Original volume = original weight of the flap (g) / muscle density (g/cm3), assuming the muscle density of 1.056 g/cm3 used for mammalian muscles (Lieber et Frieden, 2001).

4. Histology and immunohistochemistry (Studies III-V)

Eighteen muscle biopsy samples (including biopsies at two different time points in one patient) were taken from the microneurovascular muscle grafts during secondary refinement procedures (Study III). Routine histological analyses with van Gieson staining were performed on all sections both in nerve and muscle samples (Bancroft et Stevens, 1977).

The muscle samples were fixed in 4% buffered formaldehyde, embedded in paraffin, cut into 5 µm thick sections, and mounted on slides. The deparaffinized sections were pretreated for antigen retrieval (Von Boguslawsky, 1994) as shown in Table 5. Thereafter endogenous peroxidase activity was inhibited with 0.5% hydrogen peroxide in absolute methanol for 30 minutes.

Immunohistochemical stainings for muscle biopsies were performed with a Techmate 500 Immunostainer (Dako, Santa Barbara, CA, USA) using a CEM-mate visualization kit with DAB as chromogen (Dako, Glostrup, Denmark). Pretreatment with normal horse serum for 15 minutes was used to block non-specific adherence. The sections were then incubated with the diluted primary antibodies (Table 5). S-100 was used to indirectly detect the reinnervation in muscle flap by visualizing the Schwann cells, MIB-1 as a marker for cell proliferation in skeletal muscle and Myosin Heavy Chain (fast) to identify type 1 and 2 fibers. After the addition of secondary antibodies and visualization, the sections were lightly counterstained in Mayer´s hematoxylin and mounted in Aquamount (BDH Ltd, Poole, UK). In negative controls, PBS was substituted for the primary antibody.

Thirty-seven nerve graft biopsies (Study IV) were taken at the same time as the microneurovascular muscle was transferred. The nerve graft biopsies were taken from the distal end of the sural nerve graft cutting away the distal 3-4 mm to avoid the scar artefact, and the study sample was the next 5 mm of the sural nerve graft. Five healthy sural nerve samples harvested at cross-facial nerve grafting served as control nerve samples. In study V, we could use 35 nerve graft samples.

The nerve graft samples (studies IV-V) were fixed in 4% buffered formaldehyde, embedded in paraffin, cut into 5-µm thick sections, and mounted on slides. The deparaffinized sections were pretreated for antigen retrieval (Table 5). Thereafter endogenous peroxidase activity was inhibited with 0.5% hydrogen peroxide in absolute methanol for 30 min, and the sections were washed with phosphate-buffered saline (PBS). Non-specific adherence was blocked by pretreatment with normal horse serum for 15 min. The sections were then incubated with the primary antibodies (Table 5).

In study IV, Neurofilament-200 antibody reveals the number of viable axons, and S-100 protein visualizes Schwann cells (Vanstapel at al, 1986). Monoclonal antibody to human Nerve Growth Factor Receptor p75, clone 8211 (Boehringer Mannheim, Germany) was used to detect these receptors.

In study V, monoclonal anti-human endothelial cell antibody (CD-31) was used to detect the density of vascular structures in nerve graft samples and to identify the vascular structures within the nerve tissue (Table 5). The angiogenic capacity of originally avascularized nerve grafts was identified with VEGF, Flt-1 and Flk-1 (Table 5).

After addition of secondary antibodies and performance of the chromogenic reaction (AEC, Elite mono kit), the sections were lightly counterstained in Mayer’s hematoxylin and mounted in Aquamont. In negative controls, PBS was substituted for the primary antibody.

Table 5. Pretreatments and primary antibodies for immunohistochemistry (Studies III-V)

Primary antibody	Pretreatment	Dilution	Source
Ki-67 clone MIB-1 Monoclonal	citrate buffer and microwave	1:50	Immunotech, France
S-100 Polyclonal	none	1:20000	Dako, Denmark
Myosin Heavy Chain (fast) Monoclonal, clone MY-32	none	1:30000	Sigma, Germany
NF-200 Monoclonal	none	1:200	Boehringer, Mannheim, Germany
p75NGFR clone 8211 Monoclonal	protease type VIII	1:10	Boehringer, Mannheim, Germany
CD-31 Monoclonal	0.01% protease +37C	1:100	Dako, Denmark
VEGF clone G153-694 Monoclonal	citrate buffer and microwave	1:100	BD PharMingen, Europe
Flt-1 (H-225) Rabbit polyclonal	citrate buffer and microwave	1:50	Santa Cruz Biotechnology, Europe
Flk-1 (A-3) Mouse monoclonal	TrisHCl and microwave	1:100	Santa Cruz Biotechnology, Europe

4.1 Quantification of immunohistochemistry (Studies III-V)

The histological sections were analyzed by two independent observers without prior knowledge of the clinical data of the patients.

In study III, reinnervation of muscle flaps was detected indirectly immunohistochemically by visualizing Schwann cells with S-100 protein (Vanstapel et al, 1986). The density of immunopositive Schwann cells in eight randomly chosen microscopic fields in three randomly chosen sections was assessed microscopically. Due to the size of the tissue samples, all pertinent fields of the sample were covered. Each specimen was graded semiquantitatively according to the method of Madsen (Madsen et al, 1996) on the following scale from 0 to 3:

0) no S-100 immunopositivity,
1) mild S-100 immunopositivity,
2) moderate S-100 immunopositivity,
3) strong S-100 immunopositivity.

In study III, the monoclonal antibody MIB-1, which recognizes Ki-67 antigen, was used as a marker for cell proliferation in skeletal muscle. MIB-1 recognizes the antigen in formaldehyde fixed tissues and has been used to detect dividing satellite cells (Kauhanen et al, 1998b). A number of MIB-1 stained nuclei were located clearly outside the basal lamina of the muscle fiber, indicating that they were not satellite cells but belonged to a population of dividing inflammatory cells, fibroblasts, or endothelial cells seen in these areas. The latter cells were evaluated separately. In a few cases, it was difficult to define the location of the MIB-1 positive nuclei in relation to the myofiber basal lamina. To identify the basal lamina more precisely, such sections were double-stained with a modified reticulin stain according to Gordon and Sweet´s method, highlighting the basal laminae (Von Boguslawsky, 1994). A semiquantitative scoring system to classify the number of satellite cells and other mitotically active cells was used on a scale from 0 to 3:

0) no satellite cells,
1) 1-5 cells/ section,
2) 6-10 cells/ section,
3) 10+ cells/ section.

In study IV, the number of NF-200 immunopositive axons in a cross-section of the nerve graft was recorded as the percentage of the number in the control samples.

In study V, the number of vascular structures inside the nerve graft was identified and calculated in CD-31- stained cross-sections of the nerve graft. Manual calculations were made through a scoring grid amounting to 0.25 mm^2 on the section. Several pertinent fields per section were calculated to enable comparison between specimens. The results present the number of vascular structures per unit area.

Each specimen stained for p75NGFR, VEGF, Flt-1, Flk-1 (Studies IV-V) was graded semiquantitatively according to the method of Madsen (Madsen et al, 1996) on a scale from 0 to 3:

0) no antibody expression,
1) mild immunopositivity in specific antibody,
2) moderate immunopositivity,
3) strong immunopositivity in neural structures in nerve graft samples.

5. Morphometric analysis (Study III)

Morphometric analyses of muscle fiber diameter and fiber type distribution were performed. Several microscopic fields of transverse muscle sections stained with the van Gieson technique and for Myosin Heavy Chain (fast) were analyzed with a Leitz Neopromar light microscope. Images of the sections were captured to a Macintosh computer by Adobe Premiere 4.2 software, after which the smaller diameters (Dubovitz, 1985) from a minimum of 200 fibers in each case were pointed at manually with the mouse cursor and processed by Paint Pro Shop 6 (Compuserve Graphics Interchange) and Lihis (Jouko Pesonen 1998) computer programs. The muscle measurement program provides atrophy and hypertrophy factors for each sample. These factors express the number of abnormally small and abnormally large fibers in each sample. In normal adult muscle the atrophy factor has an upper normal limit of 125 and the hypertrophy factor an upper limit of 200 (Kauhanen et al, 1998a).
The extent of fatty change and fibrosis in the flaps was assessed with a semiquantitative method that graded the changes on a scale from 0 to 3.

6. Statistical analysis (Studies I-V)

In studies I –V Ordinal regression was fitted with the Statistical Package for the Social Sciences (SPSS) Polytomous Universal Model (PLUM). For univariate analysis we used the Kruskal-Wallis test (Studies I-III), Pearson and Spearman´s correlation tests (Studies II, IV,V) and Fisher´s Exact test (Studies IV- V). In multivariate analysis the complementary log-log-function (Studies I-III) and negative log-log function (Study II) were used.
Probabilities of less than 0.05 were accepted as significant (Studies I-V).
Written consent was obtained from each patient and the study was approved by the Ethics Committee of Helsinki University Hospital.

RESULTS

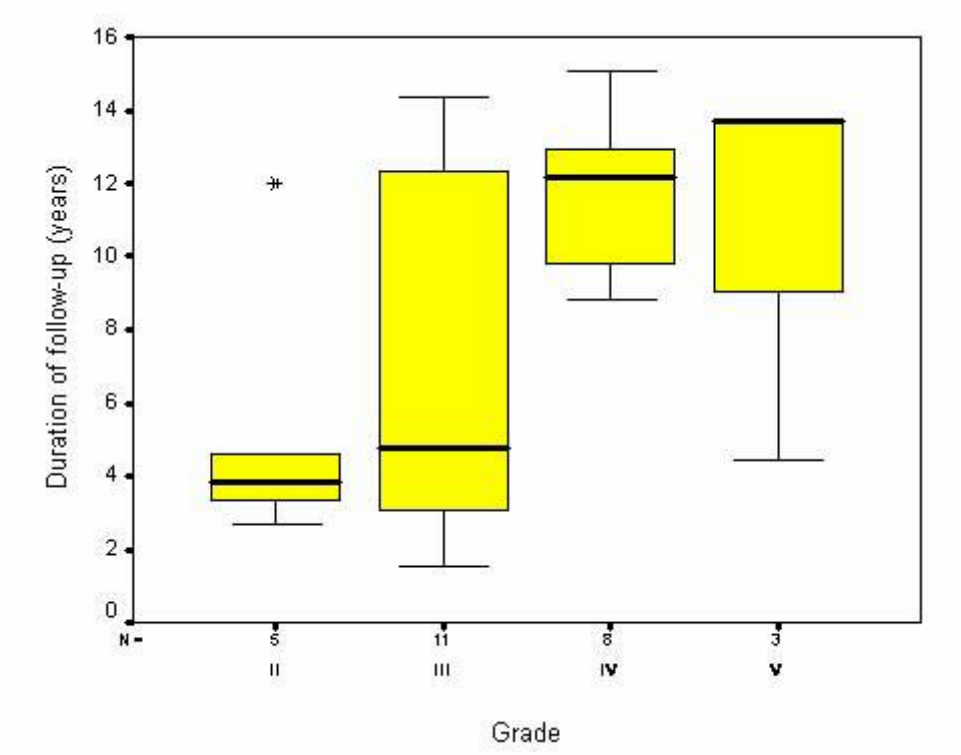
1. Long-term functional outcome of facial reanimation (Study I)

Almost two thirds of the patients achieved a result defined as only mild or moderate dysfunction (grades II-III) of facial movement. In one third of the patients, dysfunction was graded as moderately severe (grade IV) and in 10% as severe (grade V). None of the patients had retained total facial paralysis postoperatively (grade VI). The long-term functional outcome measured as the state of mimic function by House scale is summarized in Table 6**.**
In statistical analysis, the time period gone after facial reanimation made a difference on the final functional outcome, the longer the follow-up time after the muscle transplantation the weaker was the muscle function (p=0.003) as presented in Fig. 6**.**
In addition, correlations were studied between functional outcome and patients' age and sex, body mass index (BMI), smoking habits, alcohol consumption, dominant hand, aetiology and side of the palsy, duration of palsy, time interval between the two operations, duration of operation and intraoperative ischaemia, number of primary complications, and number of secondary procedures. None of these factors correlated with the functional result. None of the different donor muscles had any significant effect on final functional outcome.
The patients estimated the amount of time they had spent on mimic practice after the muscle had been transplanted. The amount of practice did not correlate with the final functional result.
Twenty-one patients (78%) thought that their quality of life had improved after the microneurovascular muscle transplantation but six (22%) considered that the reanimation had had no effect. The degree of satisfaction correlated with a good final functional result, (p= 0.008).

Table 6. Functional outcome as the postoperative state of mimic function graded according to House (House, 1983), Study I.

Grade of dysfunction	Number of patients
I Normal	0
II Mild	5
III Moderate	11
IV Moderately severe	8
V Severe	3
VI Total paralysis	0
	N=27

Figure 6. The relation between the duration of follow-up time (years) and the grading of patients' mimetic function. The solid bars indicate the median and the error bars the range.



2. Magnetic resonance imaging of microneurovascular muscle flaps (Study II)

The muscle structure appeared normal in almost half of the patients, and fat infiltrated in another six patients (Table 7). In one patient the muscle flap could not be identified, probably due to the originally small size of the flap (20 g at transfer).

Table 7. Grading of muscle structure in MRI.

Grade	Number of patients	Follow-up time after muscle transfer
I. Normal or almost normal muscle structure	6 (40%)	Mean 4y, (3-5y)
II. Muscle infiltrated with fat	6 (40%)	Mean 10y, (3-14y)
III. Severe muscle atrophy and fibrosis	2 (13%)	Mean 11y, (9-13y)
IV. Muscle could not be identified	1 (7%)	3y

The maximum thickness of the muscle flap ranged from 0.47 cm to 1.79 cm, average 0.95 cm. At the time of muscle transfer, the calculated volumes of the flaps (n=10) ranged from 18.9 to 71.0 ml, average 44.9 ml. In five patients, the weight of the muscle flap was unknown. At the time of evaluation the volumes of the flaps (n=9) ranged from 5.8 to 18.8 ml, average 10.2 ml. In seven patients, data on muscle volume both at the time of muscle transfer and at the time of MRI evaluation were available; the average loss of volume was 38.1 ml, ranging from 22.0 to 54.7 ml. In these patients, approximately 20% (range 13% to 23%) of the original volume of the muscle flap was seen in MRI, which means 80% loss of muscle volume. The average follow-up period for these patients was 3.9 years.

The muscle function was regarded as good in ten patients out of fifteen comprising mild or moderate dysfunction (House scale II to III) of facial movements. Five patients had moderately severe dysfunction (House scale IV) and none had severe dysfunction or total paralysis after reanimation.

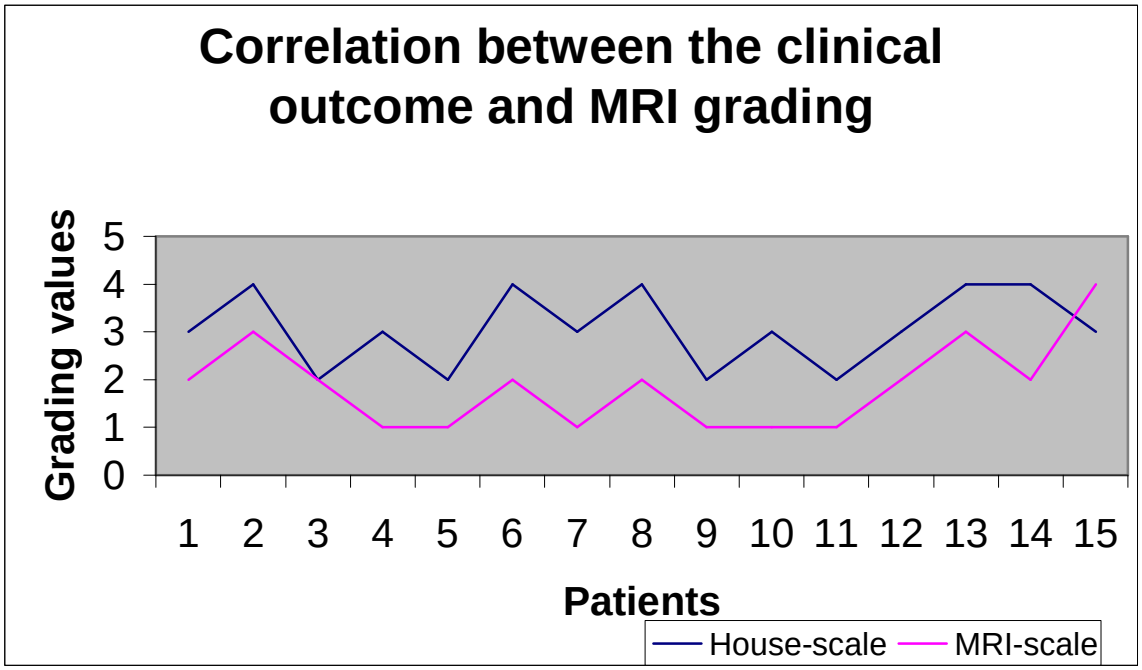
Normal muscle structure of the microneurovascular flap in MRI correlated with good mimic function after reanimation (correlation coefficient 0.59, p=0.019) as presented in Figure 7**.**

The time period elapsing between facial reanimation and evaluation was associated with the final functional outcome and the MRI grade; the longer the follow-up time after the muscle transplantation, the weaker was the muscle function (p=0.023) and the poorer was muscle structure in MRI (p=0.019).

The patients estimated the amount of time they had spent on mimic practice after the muscle transplantation. Frequent practice was associated with higher muscle volume measured in MRI. Practice, however, did not influence the MRI grade of muscle structure or the functional outcome.

Correlation studies were performed between the MRI grading of muscle structure and the muscle volume both at the time of the transplantation and at the time of evaluation, the patients' age and sex, BMI, intraoperative ischaemia and the number of secondary procedures. None of the above-mentioned factors correlated with the MRI grade or the long-term result of facial reanimation. The effect of different donor muscles was also studied statistically and no differences between the muscles were found in this patient series.

Figure 7. Correlation between the clinical outcome and MRI grading



3. Morphology of free microneurovascular muscle flaps (Study III)

The morphology of the control muscles was normal. Muscle biopsies performed 1-10 years after the second-stage operation showed a great variation in muscle fiber diameter between the 17 patients. The mean muscle fiber diameter was 38 μm (range 14-70 μm), indicating a 40% decrease compared with control values. In statistical analyses, smaller mean muscle fiber diameters of each individual correlated with prolonged intraoperative ischaemia ($p = 0.04$). Atrophy tended to be more severe in the serratus than in the other muscles (ns). The extent of fatty change and fibrosis ranged from minor to very severe as presented in Table 8.

In samples taken 1-10 years after surgery, muscle atrophy was not fiber type specific and the mean percentage of type two fibers was not different from that of control samples. Fiber type proportion was defined as the percentage of fast-twitch type 2 fibers. These showed considerable individual variation with an average value of 56%. In four patients, muscle morphology was virtually normal. In another four, the fiber type grouping typical of reinnervated muscle after denervation was detected. Statistical analysis showed that the long-term functional outcome was better in patients with a preponderance of fast fibers in their muscle grafts ($p=0.03$).

In 60% of the samples, signs of ongoing muscle regeneration through activation of satellite cells were still evident. In our patient series the proliferative activity of satellite cells declined with prolonged follow-up time (ns). The control muscle samples taken during the operation showed no proliferative activity. In some patients, proliferative activity was detected in nuclei outside the myofiber basement membrane 3-4 years after surgery. These cells included fibroblasts, inflammatory cells and endothelial cells.

After 1-10 years' follow-up, the intramuscular expression of S-100 protein indicative of reinnervation was visibile in all available samples as presented in Table 8. No correlation was found between innervation density and clinical factors in any of our patients ($p>0.05$). In control samples, muscle innervation was unaltered.

In this patient series, neither muscle morphology nor innervation were statistically affected by gender of the patient, age at the time of free muscle transfer, body mass index (BMI), smoking, time between the two operations, weight of muscle transplant, or the occurrence of primary complications.

Table 8. Fatty change, fibrosis and reinnervation of free mnv muscle flaps (Study III)

Key to scoring of fatty change and fibrosis: - none, + little, ++ moderate, +++ severe
Key to scoring of reinnervation: 0 no, 1 mild, 2 moderate, 3 strong

Patient	Fatty Change	Fibrosis	Reinnervation
1.	+	+++	specimen lacking
2.	++	++	1
3.	-	+++	2
4.	++	+++	3
5.	+	++	2
6.	+	++	2
7.	++	++	2
8.	++	+	1
9.	-	-	3
10.	+	+	1
11.	+	+++	3
12	++	+	specimen lacking
13.a	+	+	3
13.b	+	-	3
14.	-	+	3
15.	+++	+	1
16.	++	++	3
17.	++	+	specimen lacking

4. Expression of p75 Nerve Growth Factor Receptor in nerve grafts (Study IV)

In light microscopy, the grafted nerves were characterized by thickening of the epineurial and perineurial sheaths and fibrosis, notably in the endoneurial space. There was also perineurial invasion of inflammatory cells. In nerve graft biopsies, the mean number of axons expressing NF-200 amounted to 38% (range 6-81%) of that in the control samples. Axons were consistently thinner than in the control samples, and the thin axons were occasionally arranged in clusters.

Immunopositivity for S-100 indicating Schwann cells was seen in all sections. S-100 expression was stronger along the thicker axons than in samples with predominantly thin axons (ns).

In all nerve grafts studied, the expression of p75NGF receptor was clearly increased at the distal ends and was located along the axons. Table 9 illustrates the distribution of p75 expression. In our five control samples, immunopositivity for p75NGF receptor was very low or absent.

Table 9. Expression of p75NGFR in nerve grafts.

Score	Expression	Number of samples
0	none	0
1	slight	3
2	moderate	15
3	strong	19

		37

Statistical analysis showed that expression of p75NGFR was significantly lower in older than in younger people, p=0.003, Figure 8**.** A younger age for the patient also correlated with a higher number of viable axons at the distal end of the nerve graft (p=0.021).

A longer time between cross facial nerve grafting and biopsy sampling correlated strongly with a higher number of viable axons, p=0.002, Figure 9. No such correlation was noted in the time interval until biopsy and the expression of p75NGFR.

Figure 8. Correlation between the age of the patient and the expression of p75NGFR.

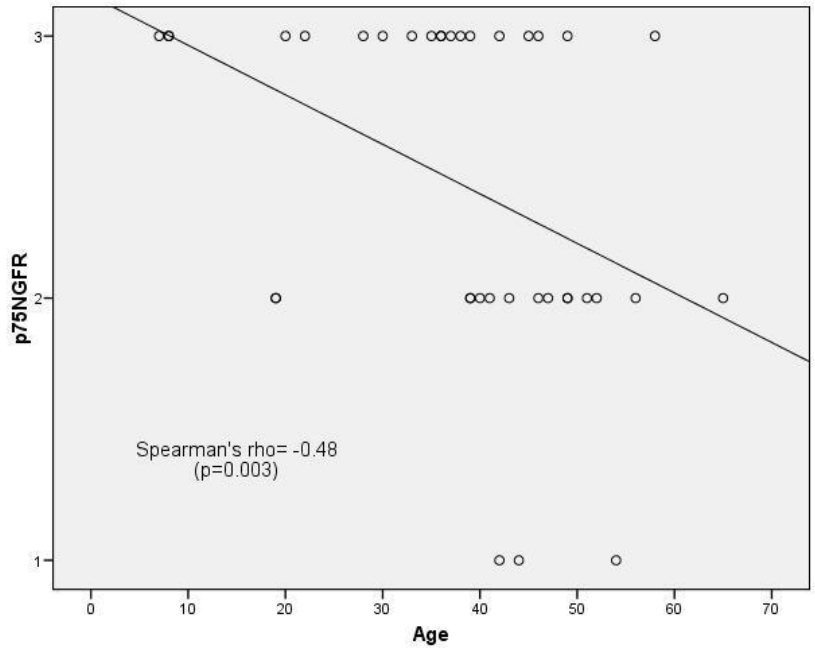
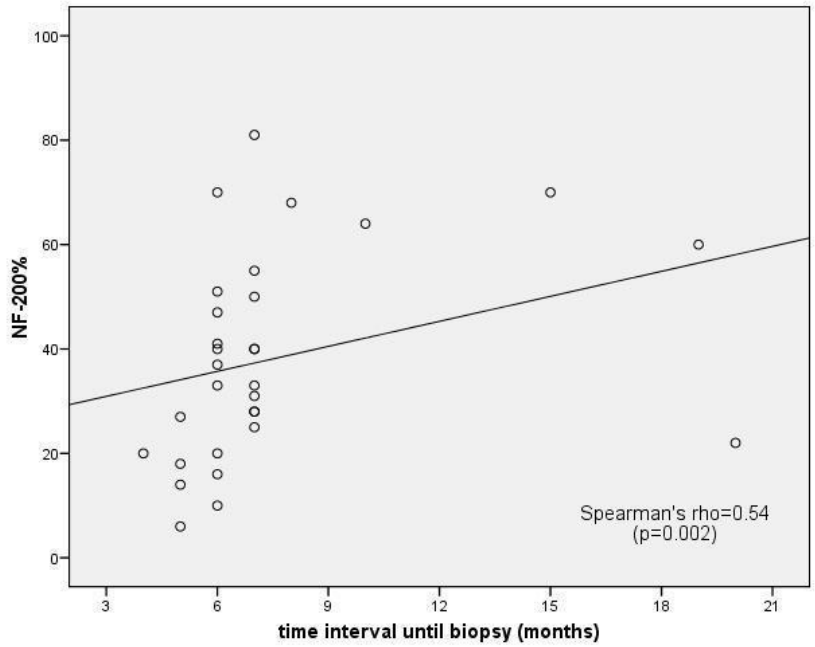
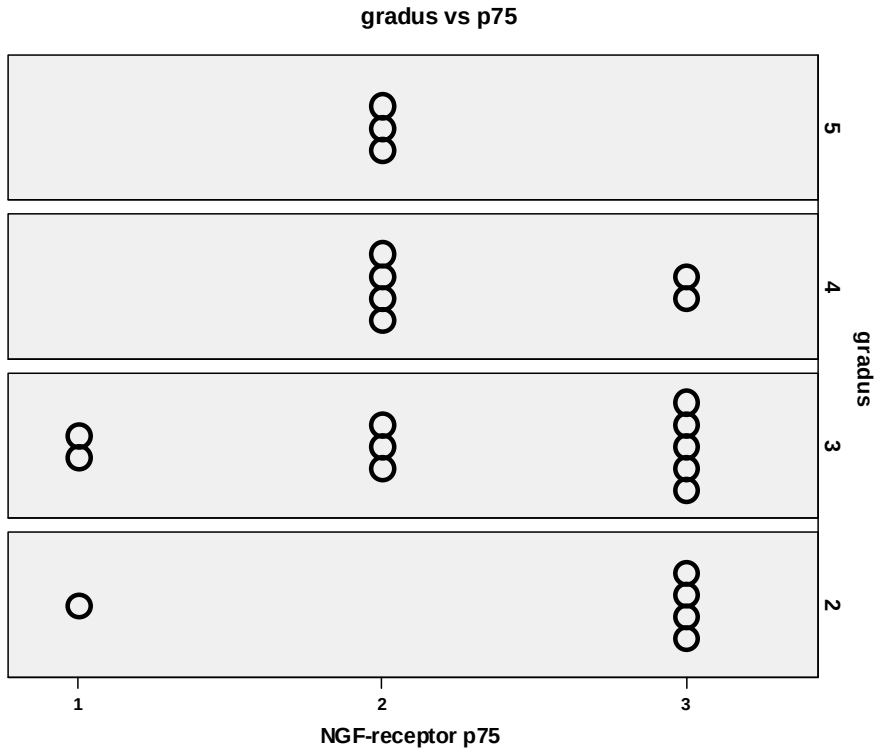


Figure 9. Correlation between the amount of viable axons and length of time until biopsy of the sural nerve graft. Number of NF-200 immunopositive axons in a cross-section of the nerve graft is recorded as the percentage of the number in the control samples.



There was an association between a high amount of viable axons in the grafted nerve and dense reinnervation of the microneurovascularly grafted muscle (correlation co-efficient 0.511, p=0.074). The expression of p75NGFR correlated with neither the number of viable axons at the distal end of the nerve graft nor the grafted muscle characteristics.
Of interest is that high expression of p75NGFR was often seen with better function of the transplanted muscle (p=0.087) as presented in Figure 10. No other of the above-mentioned factors affected the mimic function of the muscle statistically. Thus, expression of p75NGF receptor in the nerve graft is the only factor associated with good long-term mimic function of the transplanted muscle in this Study IV.

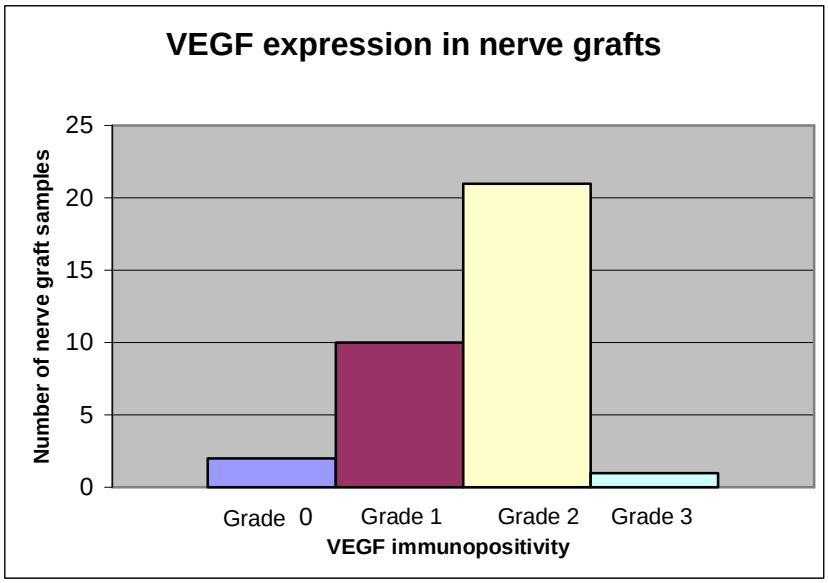
Figure 10. Relationship between the expression of p75NGFR and the function of the transplanted muscle on a grade by House (Table 2).



5. Expression of Vascular Endothelial Growth Factor and its receptors in nerve grafts (Study V)

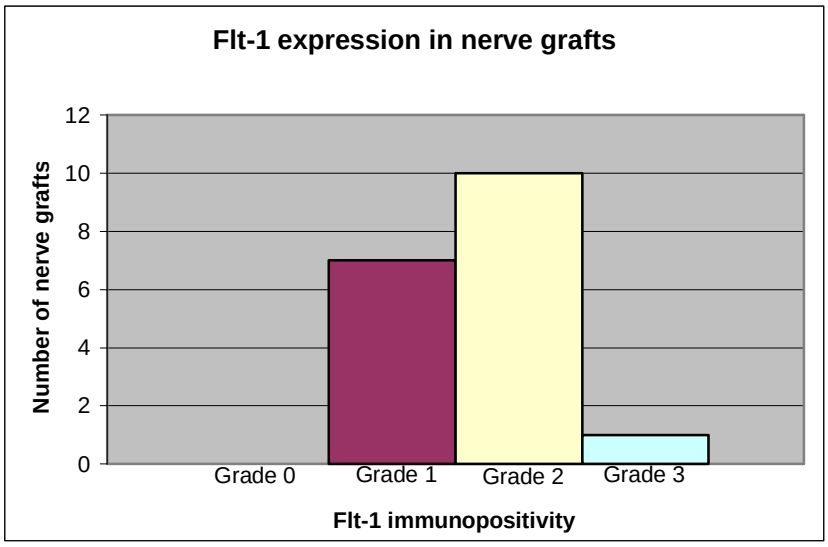
Numerous vascular structures were calculated in avascularized human nerve grafts 8 months after grafting. With CD-31 staining, we found 166 vascular structures per unit area in horizontal nerve sections, corresponding to values in control nerves. However, the variation was wide within the samples, ranging from 78 to 267 vascular structures per unit area.
VEGF was expressed more often and with higher intensity in grafted than in control nerves. Immunopositivity was most intense in axons in grafted nerves whereas in control nerves (n=3) only the myelin sheaths were immunopositive. Intensity of immunostaining for VEGF in nerve grafts (n=34) is shown in Figure 11. VEGF immunopositivity in nerve grafts and in control nerves is illustrated in original article, Study V. Endothelial cells showed high intensity of staining for VEGF in both grafted and control nerves, adding to the validity of the staining method.

Figure 11. Intensity of immunostaining for VEGF in nerve grafts



Flt-1 receptor expression was seen in axons and perineurial structures and also in endothelial cells in nerve grafts. Flt-1 receptor immunostaining intensity is presented in Figure 12. Flt-1 receptor staining in human nerve graft is shown in original Study V. In this staining our study material was limited to 18 samples. In control nerves, Flt-1 immunopositivity was mostly low in neural structures, whereas the vascular structures were clearly immunopositive.

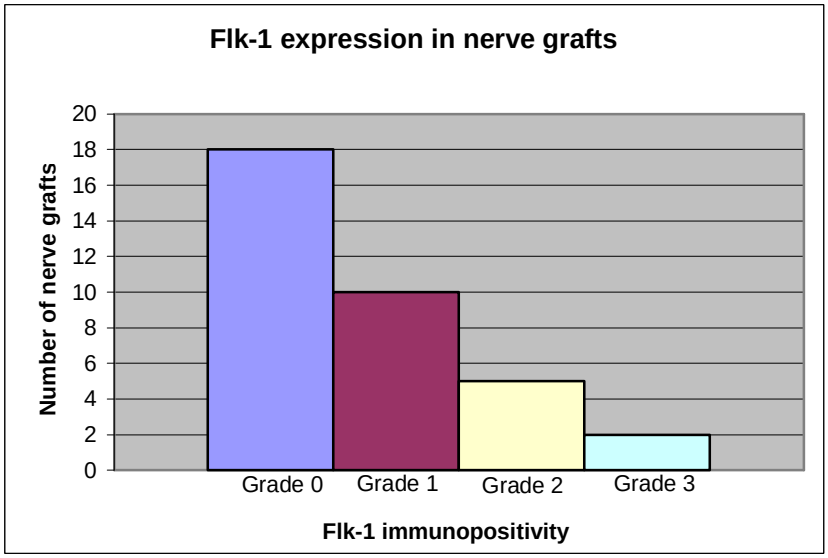
Figure 12. Intensity of immunostaining for Flt-1 in nerve grafts.



Immunopositivity for the Flk-1 receptor in neural structures (n=35) was found in only 50% of the nerve graft samples, implying that half of the samples did not have staining capacity for Flk-1 receptor. Staining intensity was mainly low, as presented in Figure 13. Immunopositivity for Flk-1 in endothelial cells was clearly visible in nerve grafts. In control nerves there was no immunopositivity in neural structures for Flk-1, whereas endothelial cells in vascular structures did stain for Flk-1.

Statistical analysis revealed a slight association between a high number of vascular structures (CD-31) and low immunopositivity in VEGF staining (ns). In our material, we found no correlation between the expression of VEGF or its receptors and viable axons (NF-200) at the distal end of the nerve graft. The expression of VEGF did not associate with the function of the grafted muscle or with the expression of p75NGFR.

Figure 13. Intensity of immunostaining for Flk-1 in nerve grafts.



DISCUSSION

Nearly thirty years of microsurgical experience in facial reanimation has contributed to our knowledge of the factors that influence the functional and aesthetic outcome after surgical treatment. However, the final functional outcome may unpredictably vary a lot among the patients.

1. Evaluation of functional outcome in facial reanimation

Several grading systems have been developed to quantify the extent of facial paralysis and the outcome of microneurovascular reanimation of the face (House, 1983; Murty et al, 1994; Chee et Nedzelski, 2000; Kahn et al, 2001; Brenner et Neely, 2004; Harrison, 2005).
Many of them have been used successfully in one study, but the problem exists when several studies are being compared each having an individual system of grading. Promising computer-assisted methods for assessing mimic function have been developed (Frey et al, 1994; Frey et al, 1999; Sargent et al, 1998), but they require technical equipments compared to the interview-based methods. In our study, facial dysfunction was measured by House's well-established standard grading system (House, 1983). The degree of dysfunction is clearly defined on a scale from 1 to 6. The patients were assessed both live during the interview and from video-recorded tapes illustrating mimic function at different sessions. Every face has a range of dimensions, and a human observer can recognise the extent of a smile not measurable in millimeters (Paletz et al, 1994). We chose the House scale because it is widely used in evaluating the loss of facial function (Novak, 2004). The consensus scoring system generated by the Fifth International Symposium on the Facial Nerve was based on the House scale (Dellon, 1992), and it is also used more recently as in studies by Manni (Manni et al, 2001) and Malik (Malik et al, 2005).
Regarding clinical outcome after facial reanimation results vary considerably. Harii provided a huge clinical data including 210 patients with cross facial nerve grafts and free muscle transfer. In this series, half of the patients achieved natural or near-natural animation. The other half had noted problems with excessive, contractions too strong or too weak, or problems with muscle attachments. (in Swartz, 2003). In other studies with different evaluation scales 42-51% of the patients had good or excellent results (Terzis et Noah, 1997; O´Brien et al, 1990). In our clinical study, 60% of the patients had good functional result at the time of evaluation, implying a quality of results similar to that of other studies. Our study material, collected in small Finnish population, will always remain lower in the number of patients than in other clinical studies conducted in large countries like USA (Terzis et Noah, 1997). In a small study, it is even harder to show any significant correlations, and in some cases, there could be more associations with larger series of patients.
When we compare the results of different papers (O´Brien et al, 1990; Sassoon et al, 1991; Terzis et Noah, 1997; Kumar et Hassan, 2002), factors such as the extent of the facial paralysis and the great variation in duration of follow-up must be taken into account. In these papers, follow-up times range from 7 months to 11 years, mean follow-up times being 2-4

years, and only 34 to 68% of the patients had complete facial palsy. In our study, all the patients had complete facial palsy. As our study was retrospective, the different patients were assessed at different time points after muscle transplantation. However, the mean follow-up period covered nearly 9 years, and was up to 15 years for some patients. These factors may be of great importance when judging the outcome, as there was a clear correlation between recent muscle transplantation and a good functional result in our study.

In other words, the older the transplant, the weaker was the muscle function. The better outcome achieved in more recent cases may have been the result of increased experience and improved technical skills on the part of the surgical team. Lack of muscle adaptation after the muscle transfer could also influence the long-term result. As stated before if there are no contractile stimuli or innervation is incomplete, the repeated attempts at regeneration will eventually exhaust satellite cells (Schmalbruch et Lewis, 1994; Yoshimura et al, 1998). Depletion of satellite cells impairs contractile function and the ability to repair tissue is lost (Jejurikar et al, 2002). In our study, however, the activation of satellite cells was associated to be time-dependent such that the longer the follow-up time, the fewer were the attempts at regeneration shown by the expression of satellite cells. It is tempting to suggest that our finding with the longer follow-up time connected with worse functional outcome can be explained, at least on one side, by the satellite cell exhaustion. There was also a tendency for association between satellite cell activity and good function of the flap. As we could not show statistically significant figures correlating these factors, the role of satellite cell exhaustion in connection to poor results after long follow-up time remains to be proved.

However, the former suggestion is confirmed by our other result as normal, regenerated muscle structure seen in MRI correlated with good function of the muscle flap. In the same study, there was a correlation between a longer follow-up time and weaker muscle function and poorer muscle structure.

The choice of muscle for microneurovascular transfer is still a matter of debate, and at least nine different muscles have been used during the search for the most appropriate one (Aviv et Urken, 1992). In our study three different muscles, serratus, gracilis and latissimus dorsi, were used.

Many authors prefer gracilis muscle as the first choice in microneurovascular muscle transfer because it has parallel muscle fibers and a constant neurovascular supply with large enough vessels to make microanastomosis safe. The muscle fibers show long amplitude of the contraction even when reduced in length, which is more important than the force when muscle is placed in the face. (Frey et Giovanoli, 2002) In a study based on cadaveric dissections, the most suitable flap was latissimus dorsi, followed by the gracilis (Bove et al, 1998).

In our study, the most severe atrophy was associated with the use of the serratus anterior muscle. It remains possible that the trend observed was due to muscle type or the fact that the serratus muscle was used in the first patients in the series, where the functional outcome was poorer than that of the later patients.

The correlation between age and regeneration of nerve and muscle is a subject of investigation. In our series, the mean age of the patients at the time of surgery was 41 years.

As most of them were middle-aged (only one was a child), we could not show the effect of age on recovery of muscle function and final functional outcome. A recent study on patients with a mean age of 22 years, however, reported a tendency for younger patients to achieve earlier functional recovery (Terzis et Noah, 1997). Similar results were obtained in a study with nerve-grafting or cranial substitution techniques, age being the most important factor influencing the functional outcome. There was a significant poorer outcome in patients older than 60 years (Guntinas-Lichius et al, 2006)

Experimental studies confirm that the youth of an animal given a graft is an important determinant of the success of regeneration of skeletal muscle (Carlson et Faulkner, 1989). The comparably older age of our patients must, therefore, be considered when evaluating our results. In a study comparing a group of children under fifteen years of age and the adults treated with two-stage microneurovascular muscle transfer, children had a significantly higher rate of the best possible grade of the function than adults. Also the first muscle contraction after surgery was detected significantly earlier in children than in adults (Ueda et al, 1998).

In our study, younger age of the patient correlated with a higher number of viable axons at the distal end of the nerve graft at the time of muscle transfer. This result is consistent with the fact that nerve regeneration is usually better in younger patients (Conley et May, 2003) although in one human cross-over nerve graft study, numbers of regenerating axons did not correlate with age (Jacobs et al, 1996). In our study, the expression of p75NGFR was significantly higher in younger than in older patients. A high level of expression of p75NGFR was weakly associated with a better function of transplanted muscle ($p=0.087$). Thus, age might be one factor influencing the functional outcome of the surgical treatment and the entire neural and muscular regeneration process.

Most of our patients 78% (21 out of 27) were satisfied with the result of their facial reanimation. Other studies have reported similar figures for satisfaction among patients (O´Brien et al, 1990; Kumar et Hassan, 2002). In the latter, 67% (10 out of 15) patients considered the result of a two-stage procedure as excellent or very good (Kumar et Hassan, 2002). As facial paralysis can be a catastrophic event for a patient, it is therefore very easy to believe that improvement of the mimic function will satisfy the patient. In our study, we could show that the degree of satisfaction correlated well with a good final result.

2. Association between functional outcome and findings in MRI

To the knowledge of the author, our article (Ylä-Kotola et al, 2005) is the first study on MRI appearance of free microneurovascular muscle flaps. In this study, we could show that a good clinical function of the microneurovascular muscle flap correlated with normal structure in MRI, implying that MRI can be used to detect even small free muscle flaps. MRI has been used for imaging facial musculature before facial nerve grafting, and there was correlation between abnormal muscle structure and poor functional outcome after nerve grafting (Kaylie et al, 2003).

Moreover, five patients with pedicled latissimus dorsi cardiomyoplasty were MRI imaged 2-4 years after myoplasty. In the MRI images, the signal intensity was similar to that of

subcutaneous fat, indicating strong fatty infiltration in muscle flap with intact vascular and nerve supply. The authors suggest that the possible explanation for the poor structure of the muscle is continuous electrical stimulation of the flap destructing muscle fibers. (Kalil-Filho et al, 1994).

It is known that denervation of the muscle causes fatty infiltration and atrophy seen in MRI and reinnervation can return the structure to normal (Uetani et al, 1993; Fleckenstein et al, 1993).

With microneurovascular muscles, reinnervation should occur, and in our study, six out of fifteen muscle flaps showed normal muscle structure, confirming that reinnervation had taken place. The other six of the muscle flaps had fatty appearance similar to that of denervated muscles or of free microvascular muscle flaps (Fleckenstein et al, 1993; Salmi et al, 1995) indicating that for some reason, reinnervation had not occurred regardless of nerve anastomosis. Worse appearance of muscle structure in MRI correlated with the weaker function of the muscle. This could indicate that failed reinnervation is at least one factor resulting in a poor functional result. That led us to a new aspect of our study; to investigate by histological methods the reinnervation of the microneurovascular muscle flaps.

3. Reinnervation of free microneurovascular muscle flaps

Two complex physiological features determine the final functional outcome after microneurovascular muscle transfer, namely, the quality of nerve regeneration and consequently, the functional recovery of the muscle transplant (Guelinckx et Sinsel, 1996).

It is well known that muscle denervation, reinnervation, and transplantation alter the expression of fast and slow muscle fibers (Oldfors et al, 1989; Frey et al, 1991; Jakubiec-Puka, 1992; Frey et al, 1998). The typical signs of reinnervation-like type grouping of the muscle can also be seen (Guelinckx et al, 1997;Giovanoli et al, 2000).

In previous studies, a significant type 1 specific atrophy and an increasing proportion of type 2 fibers in free non-innervated microvascular muscle flaps were found 9 months after surgery (Kauhanen et al, 1998a; Kauhanen et al, 2004). In an experimental study with rabbits in two-stage procedure for facial reanimation, type 2 fibers were also predominant (Rab et al, 2006).

In our study, in which the transplanted muscle was surgically neurotized with nerve anastomosis, our findings were different and no clear shift was seen in fiber type distribution. Nevertheless, adaptation of the graft to fast-twitch muscle activity favored better mimic function. We hypothesize that in those cases in which fast fibers predominated at a later time point, the choice of the donor nerve had been correct. Thus, the part of the facial nerve that normally innervates fast mimic muscles should perhaps be selected to achieve fast movements as spontaneous smile. Hence, our results are consistent with those of other studies indicating the importance of the donor nerve type in microneurovascular muscle transfer (Frey et al, 1998; Nehrer-Tairych et al, 2000).

In general, long-lasting denervation leads to degeneration of skeletal muscle fibers and to a decrease in the fiber diameter of denervated muscles (Schmalbruch et al, 1991; Sesodia et Cullen, 1991; Zhang 1997; Carpenter et Karpati, 2001; Kalliainen et al, 2002). In

experimental settings it has been demonstrated that reinnervation of a denervated muscle is essential for regaining of the initial fiber size and muscle function (Schmalbruch et al, 1991; Schmalbruch et Lewis, 1994; Cederna et al, 2001).

In our study with 18 muscle samples, the muscle fiber diameters of surgically reinnervated muscle flaps showed a 40% decrease during a follow-up period of 1-10 years. In a study of a few cases of facial reanimation, however, there was no significant decrease in fiber diameter despite similar follow-up times (Yoshimura et al, 1998). Whether the difference between their results and ours could be attributed to differences in functional recovery of the transplanted muscles remains unknown, as no functional grading was reported in Yoshimura's study (Yoshimura et al, 1998). Moreover, the decrease in muscle fiber diameters noted in our series was similar to that observed by Frey in seven patients (Frey et al, 1991).

Our present results show that 1-10 years after microneurovascular muscle transfer, intramuscular expression of S-100 protein indicative to some extent of reinnervation was present in all muscle biopsy samples studied. On the basis of indirect morphological features, Yoshimura at al. hypothesized that reinnervation had failed to occur in a few of their cases of human microneurovascular muscle transfer, and that the flap had therefore atrophied (Yoshimura et al, 1998). In our study, however, despite obvious muscle innervation, muscle morphology was not fully restored in all cases. Moreover, the density of neural structures correlated with neither atrophy, function, nor any other particular parameters in our patient series. In experimental setting, reinnervated microvascular flaps showed a considerable amount of muscle atrophy regardless of good reinnervation. Thus, it seems that muscle fiber size is affected by factors other than innervation alone (Kostakoglu et al, 1995; Goldspink et al, 1995). In general, muscle fibers kept at less than resting length or inactive will eventually atrophy (Wichester et Goneyea, 1992; Roy et al, 1992). In addition, in a previous study on the application of free muscle flaps to the lower limb, in which the nerve was not re-connected, expression of S-100 and PGP 9.5 had disappeared at 2 weeks. However, by 9 months after surgery, it had spontaneously reappeared in two thirds of patients, indicating spontaneous "intrinsic" neurotization of flaps as part of the regenerative regime (Kauhanen et al, 2003).

In our study, control biopsies taken during the muscle transfer showed no proliferative activity in satellite cells. Satellite cell activation is known to occur after denervation (Oldfors et al, 1989; Gosselin et al, 1994; Kuschel et al, 1999; Yoshimura et Harii, 1999) and muscle transplantation (Carlson et Faulkner, 1988; Carlson et Faulkner, 1989). In our study, 60 % of the muscle samples showed satellite cell activity indicating that regeneration takes place even several years after microneurovascular muscle transfer even though reinnervation seems to be completed. This finding is compatible with earlier observations on the continuation of muscle regeneration years after muscle transfer (Yoshimura et al, 1998). If there are no contractile stimuli or innervation is incomplete, the repeated attempts at regeneration will eventually exhaust satellite cells (Schmalbruch et Lewis, 1994; Yoshimura et al, 1998). Depletion of satellite cells impairs contractile function and the ability to repair tissue is lost (Jejurikar et al, 2002). Opinions differ as to whether reinnervation promotes myogenic potential or whether regenerating muscle fibers actually enhance neural ingrowth (Carlson et Faulkner, 1988). In our study, even if the muscle flap was reinnervated there were signs of continuous muscle

regeneration. This might indicate that reinnervation has occurred soon after transplantation and later, when biopsies are taken at the time-point convenient to refinement surgery, there might have been many contributing factors other than reinnervation alone. It can be summarized that although the free microneurovascular muscle flaps seem to be reinnervated, the morphology is not unaltered and there are still attempts at muscle regeneration going on. The question arises if nerve regeneration through the nerve graft is good enough to maintain the muscle structure.

4. Immunohistochemical findings in human nerve grafts

In our study with 37 nerve graft specimen, the average proportion of viable fibers at the distal end of the nerve graft was about 40% of that in control samples and thin nerve fibers were characterized by endoneurial fibrosis although showing individual variation. In earlier human studies, the results were similar to ours in terms of fibrosis and diminished number of viable nerve fibers grown to the distal end of the grafted nerve (Vedung et Olsson, 1982; Frey et al, 1991; Frey et al, 1996). The number of regenerated axons did not correlate with functional outcome as also shown earlier (Frey et al, 1991; Frey et al, 1996).

The use of vascularized nerve grafts has been explained by better survival of neural tissue (Koshima et Harii, 1985; Schultes et al, 2001). Our nerve grafts were nonvascularized sural nerve grafts, but they showed good vascularization regardless of fibrosis.

In an experimental acellular nerve conduit model, it has been shown that neovascularization preceded axonal regeneration. Newly formed axons and Schwann cells never exceeded the area of vascularization (Hobson et al, 1997). Although vascularization contributes to nerve regeneration, the regeneration process certainly involves many other factors as well (Hobson et al, 1997; Sondell et al, 2000). In our material, the axon number is reduced even though vascularity is good: it remains obscure whether neovascularization within the nerve graft occurred too late to have a positive effect on nerve regeneration, as the timepoint when a sample was taken was constant in our study. We therefore hypothesize that if there were less vascularity, the nerve would be even more fibrous.

It has been suggested that VEGF is one of the factors contributing not only to vascularity but also to nerve regeneration (Carmeliet et Storkebaum, 2002; Storkebaum et al, 2004). Two similar experimental models showed that the effect of added VEGF in nerve regeneration was dose-dependent (Sondell et al, 1999b; Hobson et al, 2000). Apparently only the vascular structures and Schwann cells were stimulated with lower concentration of VEGF (Sondell et al, 1999b). Higher concentration of VEGF stimulated also axonal regeneration (Hobson et al, 2000). In our material, we discovered good vascularity but moderate nerve regeneration. We do not know the amount of VEGF expression in the graft immediately after nerve transfer. It may have been too low to stimulate the growth of neural structures effectively but high enough to grow the vascular structures. This hypothesis is supported by our finding that there was no statistical correlation between the expression of VEGF and viable axons at the distal end of the nerve graft.

The expression of VEGF and Flt-1 receptor was distibuted quite similarly in our study, as the percentage of moderate immunopositivity (grade 2) was 62% and 55%, respectively. The other receptor, Flk-1, was expressing itself much weaker, only 14% had grade 2 immunopositivity. It has been proposed that the neurotrophic effect of VEGF is mostly mediated through Flk-1 (Zhang et al, 2003). As we could not show causality between the expression of VEGF and neurotrophism, one explanation could be the mild immunopositivity of Flk-1 receptor in our data.
The expression of VEGF was not associated with the expression of p75NGFR at the distal ends of the nerve graft. If growth factors and their receptors are needed at certain accurate time points to function correctly in nerve regeneration, it is difficult to estimate statistical significances in our human material, as clinical rules require us to take samples at a constant time-point.

In our series, we could show p75NGF receptor expression in every nerve graft sample. In most cases, the expression was strong, grade 3 percentage being 51%. In an experimental setting, all the regenerating spinal axons expressed p75NGFR, while none of the motoneurons that failed to regenerate expressed p75NGFR. Hence, the authors suggest that p75NGFR could be a marker of ongoing axonal regeneration (Wu et al, 2004). Our results imply that high expression of p75NGFR in nerve grafts at the time of muscle transfer is favourable for the regeneration process, but further studies are needed in this field.
There was no correlation between p75NGFR and the number of viable axons in our data. This could mean that p75NGFR is expressed during a limited time period of the nerve regeneration process. In an experimental work, Liu et al. describe a decline in NGF and p75NGFR expression at a wound when axonal sprouts had reached the distal segment (Liu et al, 1995). In our study, p75NGFR expression was monitored at only one time point owing to obvious limitations in human tissue sampling. P75NGFR also co-operates with other receptors and neurotrophins in a complex way (Schor, 2005; Ferri et al, 2002) so that further research is warranted to immunostain different receptors combined with neurotrophins.
Our study shows that VEGF, Flk-1, Flt-1 and p75NGFR are expressed in human nerve grafts and that the pattern of expression is unlike that in control nerves. This might indicate that regeneration is continuing in nerve grafts at the moment of muscle transfer as the growth factors and the receptors are still present.

5. Future prospects

In present study, we wanted to evaluate the results of the facial reanimation surgery as well as nerve and muscle regeneration within the same human model. As discussed above, many factors contribute to the results of the study due to the obvious limitations in human experiments and tissue sampling. However, further research in this field is essential in order to gain knowledge about nerve and muscle regeneration. I hope that in near future, with increased knowledge of growth factors working together in regeneration process, patients with severe illnesses demanding microneurovascular free muscle flaps will also benefit from this kind of research. In facial reanimation model, both nerve and muscle regeneration and brain plasticity are important factors influencing the outcome of the surgery. It is of extensive importance that the actual in vivo situation in human tissue has been described when planning growth factor treatments. Further research by our group will elucidate the changes in brain function after reanimation.

CONCLUSIONS

1. Most of the patients achieved good functional result after two-stage reanimation, and they were satisfied with the result. Mimic function, however, was poorer with longer postoperative follow-up time.

2. The volume of the free microneurovascular muscle flaps declined significantly comparing the initial volume of the flap. There was a correlation between good mimic function and normal muscle structure seen in MRI.

3. Muscle fiber diameter was diminished by 40% compared to control values despite the fact that all samples were reinnervated. Proliferative activity of satellite cells was seen in 60% of the samples but it tended to decline with prolonged follow-up time. Severe muscle atrophy correlated with prolonged intraoperative ischaemia suggesting that the duration of ischaemia should be kept in minimum.

4. Although all the nerve grafts were well vascularized, there was fibrosis in nerve grafts with fewer and thinner axons compared to control nerves.

5. P75NGFR was expressed in every nerve graft in axons whereas in control nerves, the expression of p75NGFR was low or absent. The expression of p75NGFR was higher in younger than older patients. VEGF and Flt-1 were strongly expressed in nerve grafts, but in control nerves the immunopositivity was low. This indicates that these growth factors and receptors are present in human nerve grafts during the regeneration process.

6. Mimic function was better in patients with a preponderance of fast fibers in muscle transplants. A high expression of p75NGFR was often seen in patients with better mimic function (ns). The number of viable axons in the distal ends of the nerve grafts did not correlate with the function of the transplanted microneurovascular muscle.

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REFERENCES

Anderl, H. Reconstruction of the face through cross-face-nerve transplantation in facial paralysis. Chir Plastica 2: 17-46, 1973.

Aviv JE, Urken ML. Management of the paralyzed face with microneurovascular free muscle transfer. Arch Otolaryngol Head Neck Surg 118: 909-912, 1992.

Baker DC, Conley J. Regional muscle tranplantation for the rehabilitation of the paralyzed face. Clin Plast Surg 6: 317-31, 1979.

Bancroft JD, Stevens A. A theory and practice of histological techniques, 1st edn, p 40. Churchill Livingstone, Edinburgh, UK, 1977.

Bendszus M, Koltzenburg M, Wessig C, Solymosi L. Sequential MR imaging of denervated muscle: experimental study. Am J Neuroradiol 23: 1427-31, 2002.

Bernat I, Vitte E, Lamas G, Soudant J, Wiler JC et Tankere F. Related timing for peripheral and central plasticity in hypoglossal-facial nerve anastomosis. MuscleNerve 33: 334-41, 2006.

Bodine-Fowler S. Skeletal muscle regeneration after injury: An Overview. J Voice 8: 53-62, 1994.

Bove A, Chiarini S, Dándrea V et al. Facial nerve plasy: which flap? Microsurgical, anatomical, and functional considerations. Microsurg 18: 286-289, 1998.

Boyd JG, Gordon T. The neurotrophin receptors, trkB and p75, differentially regulate motor axonal regeneration. J Neurobiol 49: 314-325, 2001.

Boyd JG, Gordon T. Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. Mol Neurobiol 27: 277-324, 2003.

Brenner MJ, Neely JG. Approaches to grading facial nerve function. Semin Plast Surg 18(1): 13-21, 2004.

Byrne PJ. Importance of facial expression in facial nerve rehabilitation. Curr Opin Otolaryngol Head Neck Surg 12(4): 332-35, 2004.

Campion DR. The muscle satellite cell, a review. Int Rev Cytol 87: 225-51, 1984.

Carlson BM, Faulkner JA. The regeneration of skeletal muscle fibers following injury: a review. Med Sci Sports Exerc 15: 187-198, 1983.

Carlson BM, Faulkner JA. Reinnervation of long-term denervated rat muscle freely grafted into an innervated limb. Exp Neurol 102: 50-55, 1988.

Carlson BM, Faulkner JA. Muscle transplantation between young and old rats: age of host determines recovery. Am J Physiol 256: 1262-1266, 1989.

Carmeliet P, Storkebaum E. Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. Cell Developm Biology, 13: 39-53, 2002.

Carpenter S, Karpati G. Pathology of skeletal muscle, p 317. Oxford University Press, New York, 2001.

Cederna PS, Kalliainen LK, Urbanchek MG, Rovak JM, Kuzon WM Jr. Donor muscle structure and function after end-to-side neurorrhaphy. Plast Reconstr Surg 107(3): 789-96, 2001.

Chee GH, Nedzelski JM. Facial nerve grading systems. Facial Plast Surg 16(4): 315-24, 2000.

Choi D, Dunn LT. Facial nerve repair and regeneration: an overview of basic principles for neurosurgeons. Acta Neurochir 143: 107-14, 2001.

Chong J, Chan LL, Langstein HN, Gingsberg LE. MR Imaging of the muscular component of myocutaneous flaps in the head and neck. Am J Neuroradiol 22: 170-74, 2001.

Chuang D. Technique evolution for facial paralysis reconstruction using functioning free muscle transplantation - experience of Chang Gung Memorial Hospital. Clin Plast Surg 29: 449-459, 2002.

Conley J, Baker DC. Hypoglossal-facial nerve anastomosis for reinnervation of the paralyzed face. Plast Reconstr Surg 63: 3- 72, 1979.

Conley J, May M. Perspective in Facial reanimation. In Facial Paralysis, Rehabilitation Techniques, pp 1-20. Eds May M, Schaitkin B. Thieme, New York, 2003.

Cooper RR. Alterations during immobilisation and regeneration of skeletal muscle in cats. J Bone Joint Surg 54-A: 919-953, 1972.

Cote C, Faulkner JA. Characteristics of motor units in muscles of rats grafted nerves intact. Am J Physiol 250: C828, 1986.

Dechant G, Tsoulfas P, Parada LF, Barde Y-A. The neurotrophin receptor p75 binds neurotrophin-3 on sympathetic neurons with high affinity and specificity. J Neurosci 17: 5281-87, 1997.

Dechant G, Barde Y-A. The neurotrophin receptor p75: novel functions and implications for diseases of the nervous system. Nat Neurosci 5: 1131-36, 2002.

Dellon AL, Restoration of facial nerve function: An approach for the Twenty-First century. Neurosurg Quart 2(3): 199-222, 1992.

Dooms GC, Hricak H, Margulis AR, de Greer G. MR imaging of fat. Radiology 158: 51-54, 1986.

Drobnick, cited by Sawicki B. In the Status of Neurosurgery, p 189. Ed Chepault. J Rueff, Paris, 1902.

Dubowitz V. Muscle Biopsy. A Practical Approach. 2nd ed, p 82-94. Bailliere Tindall, London, UK, 1985.

Ernfors P, Henschen A, Olson L, Persson H. Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. Neuron 2: 1605-13, 1989.

Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161: 851-58, 1989.

Ferri C, Moore F, Bisby M. Effects of facial nerve injury on mouse motoneurons lacking the p75 low-affinity neurotrophin receptor. J Neurobiol 34: 1-9, 1998.

Ferri C, Bisby M. Improved survival if injured sciatic nerve Schwann cells in mice lacking the p75 receptor. Neurosci Lett 272: 191-94, 1999.

Ferri CC, Ghasemlou N, Bisby MA, Kawaja MD. Nerve growth factor alters p75 neurotrophin receptor-induced effects in mouse facial motoneurons following axotomy. Brain Res 950(1-2): 180-85, 2002.

Fischbein NJ, Kaplan MJ, Jackler RK, Dillon WP. MR Imaging in two cases of subacute denervation change in the muscles of facial expression. Am J Neuroradiol 22: 880-84, 2001.

Fleckenstein JL, Watumull D, Conner KE et al. Denervated human skeletal muscle: MR imaging evaluation. Radiology 187: 213-18, 1993.

Frey M, Gruber H, Havel M, et al. Experimental free muscle transplantation with microneurovascular anastomoses. Plast Reconstr Surg 71: 689-702, 1983.

Frey M, Happak W, Girsch W, Bittner RE, Gruber H. Histomorphometric studies in patients with facial palsy treated by functional muscle transplantation. New aspects of the surgical consept. Ann Plast Surg 26: 370-79, 1991.

Frey M, Koller R, Gruber I. et al. Time course of histomorphometric alterations in nerve grafts without connection to a muscle target organ: An experimental study in sheep. J Reconstr Microsurg 8: 345-57, 1992.

Frey M, Jenny A, Giovanoli P, Stussi E. Development of a new documentation system for facial movements as a basis for the international registry for neuromuscular reconsruction in the face. Plast Reconstr Surg 93: 1334-1349, 1994.

Frey M, Koller R, Liegl C, Happak W, Gruber H. Role of a muscle target organ on the regeneration of motor nerve fibres in long nerve grafts: a synopsis of experimental and clinical data. Microsurg 17: 80-88, 1996.

Frey M, Giovanoli P, Meuli-Simmen C: The qualification of different free muscle transplants to reconstruct mimic function. Plast Reconstr Surg 101: 1774-83, 1998.

Frey M, Giovanoli P, Gerber H, Slameczka M, Stussi E. Three-dimensional video analysis of facial movements: a new method to assess the quantity and quality of the smile. Plast Reconstr Surg 104: 2032-2039, 1999.

Frey M, Giovanoli P. The three-stage concept to optimize the results of microsurgical reanimation of the paralyzed face. Clin Plast Surg 29: 461-482, 2002.

Frey M, Giovanoli P, Michaelidou M. Functional upgrading of partially recovered facial palsy by cross-face nerve grafting with distal end-to-side neurorrhaphy. Plast Reconstr Surg 117: 597-608, 2006.

Frostick SP, Yin Q, Kemp GJ. Schwann cells, neurotrophic factors, and peripheral nerve regeneration. Microsurg 18: 397-405, 1998.

Gillies H: Experiences with fascia lata grafts in the operative treatment of facial paralysis. Proced R Soc Med 27:1372, 1934.

Giovanoli P, Koller R, Meuli-Simmen C, Rab M, Haslik W, Mittlböck M, Meyer V, Frey M. Functional and morphometric evaluation of end-to-side neurorrhaphy for muscle reinnervation. Plast Reconstr Surg 106: 383-92, 2000.

Goldspink DF, Cox VM, Smith SK, Eaves LA, Osbaldeston NJ, Lee DM, Mantle D. Muscle growth in response to mechanical stimuli. Am J Physiol 268: 288-97,1995.

Gosselin LE, Brice G, Carlson B, Prakash YS, Sieck GC: Changes in satellite cell mitotic activity during acute period of unilateral diaphragm deneravation. J Appl Physiol 77: 1128-34, 1994.

Gray H. Anatomy, descriptive and surgical, p.742. Ed Pick TP, Howden R. Gramercy books, New York, 1977.

Grounds M. Towards understanding skeletal muscle regeneration. Pathol Res Pract 187: 1-22, 1991.

Gschwendtner A, Liu ZQ, Hucho T, Bohatschenk M, Kalla R, Dechant G, Raivich G. Regulation, cellular localization, and function of the p75 neurotrophin receptor during the regeneration of facial motoneurons. Mol Cell Neurosci 24: 307-22, 2003.

Guelinckx PJ, Faulkner JA, Essig DA. Neurovascular anastomosed muscle grafts in rabbits: Functional deficits result from tendon repair. MuscleNerve 11: 745-51, 1988.

Guelinckx P, Sinsel N. Muscle transplantation for reconstruction of a smile after facial paralysis past, present and future. Microsurg 17: 391-401, 1996.

Guelinckx P, Sinsel N, Dom R. Contemporary muscle morphology as related to nerve pathology. In Management of peripheral nerve problems, pp 663-67. Ed. Omer, Spinner and Van Beek. Saunders, 1997.

Guntinas-Lichius O, Streppel M, Stennert E. Postoperative functional evaluation of different reanimation techniques for facial nerve repair. Am J Surg 191:61-67, 2006.

Harii K, Ohmori K, Torii, S. Free gracilis muscle transplantation with microneurovascular anastomoses for the treatment of facial paralysis. Plast Reconstr Surg 57: 133- 43, 1976.

Harii K. Microneurovascular free muscle transplantation for reanimation of facial paralysis. Clin Plast Surg 6: 361-75, 1979.

Harii K, Asato H, Yoshimura K, Sugawara Y, Nakatsuka T, Ueda K. One-stage transfer of the Latissimus dorsi muscle for reanimation of a paralyzed face: a new alternative. Plast Reconstr Surg 102: 941-51, 1998.

Harrison DH. The pectoralis minor vascularized muscle graft for the treatment of unilateral facial palsy. Plast Reconstr Surg 75: 206-13, 1985.

Harrison DG. The treatment of unilateral and bilateral facial palsy using free muscle transfers. Clin Plast Surg 29: 539-49, 2002.

Harrison DG. Surgical correction of unilateral and bilateral facial palsy. Postgrad Med J 81: 562-67, 2005.

Hata Y, Yano K, Matsuka K, Ito O, Matsuda H, Hososkawa K. Treatment of chronic facial palsy by transplantation of the neurovascularized free rectus abdominis muscle. Plast Reconstr Surg 86: 1178-1187, 1990.

Hefti F, Hartikka J, Salvatierra A, Weiner WJ, Mash DC. Localization of nerve growth factor receptors in cholinergic neurons of the human basal forebrain. Neurosci Lett 69(1): 37-41, 1986.

Hobson MI, Brown R, Green CJ, Terenghi G. Inter-relationships between angiogenesis and nerve regeneration: a histochemical study. Brit J Plast Surg 50: 125-131, 1997.

Hobson MI, Green CJ, Terenghi G. VEGF enhances intraneural angiogenesis and improves nerve regeneration after axotomy. J Anat 197: 591-605, 2000.

House J. Facial nerve grading systems. Laryngoscope 93: 1056-69, 1983.

Hudgins PA, Burson JG, Gussack GS, Grist WJ. CT and MR appearance of recurrent malignant head and neck neoplasms after resection and flap reconstruction. Am J Neuroradiol 15: 1689-94, 1994.

Islamov RR, Chintalgattu V, Pak ES, Katwa LC, Murashov AK. Induction of VEGF and its Flt-1 receptor after sciatic nerve crush injury. Neuro Rep 15: 2117-21, 2004.

Jacobs JM, Laing JHE, Harrison DH. Regeneration through a long nerve graft used in the correction of facial palsy. Brain 119:271-79, 1996.

Jakubiec-Puka A. Changes in myosin and actin filaments in fast skeletal muscle after denervation and self-innervation. Comp Biochem Physiol 102A: 93-99, 1992.

Jejurikar SS, Marceleo CL, Kuzon WM Jr. Skeletal muscle denervation increases satellite cell susceptibility to apoptosis. Plast Reconstr Surg 110: 160-68, 2002.

Jiang H, Guo ET, Ji ZL, Zhang ML, Lu V. One-stage microneurovascular free abductor hallucis muscle transplantation for reanimation of facial paralysis. Plast Reconstr Surg 96(1): 78-85, 1995.

Kahn J, Gliklich, R, Boyev K, Stewart M, Metson R, McKenna M. Validation of a patient-graded instrument for facial nerve paralysis: The FaCE Scale. Laryngoscope 111: 387-98, 2001.

Kalantarian B, Rice DC, Tinagco DA, Terzis JK. Gains and losses of the XII-VII component of the “babysitter” procedure. A morphometric analysis. J Reconstr Microsurg 14(7): 459-71, 1998.

Kalil-Filho R, Bocchi E, Weiss R, Rosemberg L, Bacal F, Moreira L et al. Magnetic resonance imaging evaluation of chronic changes in latissimus dorsi cardiomyoplasty. Circulation 90: 102-06, 1994.

Kalliainen LK, Sameer S, Jejurikar MD, Liang LW, Urbanchek MG, Kuzon WM Jr. A specific force deficit exists in skeletal muscle after partial denervation. MuscleNerve 25(1): 31-38, 2001.

Kanerva M, Pitkäranta A. Perifeerinen kasvohalvaus. Duodecim 122: 2267-74, 2006.

Kauhanen S, Leivo I, Pettilä M et Michelsson JE. Recovery of skeletal muscle after immobilization of rabbit hindlimb. APMIS 104: 797-804, 1996.

Kauhanen S, Salmi A, von Boguslawsky K, Leivo I, Asko-Seljavaara S. Muscle fiber diameter and muscle type distribution following free microvascular muscle transfer: a prospective study. Microsurg 18: 137-44, 1998a.

Kauhanen S, von Boguslawsky K, Michelsson J-E, Leivo I. Satellite cell proliferation in rabbit hindlimb muscle following immobilization and remobilization. Acta Neuropathol 95: 165-70, 1998b.

Kauhanen S, Salmi A, von Boguslawsky K, Asko-Seljavaara S, Leivo I. Satellite cell proliferation, reinnervation, and revascularization in human free microvascular muscle flaps. J Surg Res 115(2): 191-99, 2003.

Kauhanen MS, Lorenzetti F, Leivo IV, Tukiainen E, Asko-Seljavaara SL. Long-term morphometric and immunohistochemical findings in human free microvascular muscle flaps. Microsurg 24(1): 30-38, 2004.

Kaylie D, Wax M, Weissman J. Preoperative facial muscle imaging predicts final facial function after facial nerve grafting. AJNR 24:326-30, 2003.

Klebuc M, Shenaq SM. Donor nerve selection in facial reanimation surgery. Semin Plast Surg 18, 1: 53-59, 2004.

Klein R, Nanduri V, Shuqian J, Lamballe F, Tapley P et al. The trkB Tyrosine Protein Kinase is a receptor for Brain-Derived Neurotrophic Factor and Neurotrophin-3. Cell 66: 395-403, 1991.

Koller R, Frey M, Meier U, Liegl Ch, Gruber H, Meyer VE. Fiber regeneration in nerve grafts without connection to a target muscle. An experimental study in rabbits. Microsurg 14: 516-26, 1993.

Koshima I, Harii K. Experimental study on vascularized nerve grafts. Morphometric study of axonal regeneraton of nerves transplanted into silicone tubes. Ann Plast Surg 14(3): 235-43, 1985.

Koshima I, Moriguchi T, Soeda S, Hamanaka T, Tanaka H, Ohta S. Free rectus femoris muscle transfer for one-stage reconstruction of established facial paralysis. Plast Reconstr Surg 94: 421-30, 1994.

Kostakoglu N, Terenghi G, Manek S, Batchelor AG, Polak JM, Green CJ. Reinnervation and neovascularization in prefabricated free muscle flaps. Microsurg 16: 388-95, 1995.

Kumar PAV, Hassan KM. Cross-face nerve graft with free-muscle transfer for reanimation of the paralyzed face: a comparative study of the single-stage and two-stage procedures. Plast Reconstr Surg 109: 451-62, 2002.

Kuschel R, Yablonka-Reuveni Z, Bornemann A. Satellite cells on isolated myofibers from normal and denervated adult rat muscle. J Histochem Cytochem 47: 1375-84, 1999.

Kuzbari R, Worseg A, Burggasser G, Schlenz I, Kuderna C, Vinzenz K, Gruber H, Holle J. The external oblique muscle free flap. Plast Reconst Surg 99(5):1338-45, 1997.

Kääriäinen M. Adhesion and Biomechanics in Regenerating Skeletal Muscle. Acta Universitatis Tamperensis 796. Tampereen yliopistopaino, Tampere, 2001.

Labbe D, Huault M. Lengthening temporalis myoplasty and lip reanimation. Plast Reconstr Surg 105: 1289-1297, 2000.

Lamminen AE. Magnetic resonance imaging of primary skeletal muscle diseases: patterns of distribution and severity of involvement. Brit J Radiol 63: 946-50, 1990.

Lee EI, Hurvitz KA, Evans GRD, Wirth GA. Cross-facial nerve graft: past and present. JPRAS 61:250-56, 2008.

Levi-Montalcini R, Hamburger V. Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J Exp Zool 116(2): 321-61, 1951.

Lieber RL, Frieden J. Clinical significance of skeletal muscle architecture. Clin Orthopaed Relat Res 383: 140-51, 2001.

Liu HM, Lei HY, Kao K-P. Correlation between NGF levels in wound chamber fluid and cytological localization of NGF and NGF receptor in axotomised rat sciatic nerve. Exp Neurol 132: 24-32, 1995.

Madsen JE, Wang J-S, Hukkanen M, Nordsletten L, Konttinen YT, Santavirta S, Polak JM, Aspenberg P. Sensory nerve ingrowth during bone graft incorporation in the rat. Acta Orthop Scand 67: 217-20, 1996.

Malik T, Kelly G, Ahmed A et al. A comparison of surgical techniques used in dynamic reanimation of the paralyzed face. Otol Neurotol 26:284-91, 2005.

Manktelow R, Zuker R, McKee N. Functioning free muscle transplantation. J Hand Surg Am 9A: 32-39, 1984.

Manni J, Beurskens C, Van de Velde C et al. Reanimation of the paralyzed face by indirect hypoglossal-facial nerv anastomosis. Am J Surg 182:268-73, 2001.

Mathes SJ, Hansen SC. Flap classification and applications. In Plastic Surgery, Vol I, pp 365-482.
Ed Mathes SJ. Saunders, Philadelphia, 2006.

May M. Nerve Repair. In Facial Paralysis, Rehabilitation Techniques, pp 21-59. Eds May M, Schaitkin B. Thieme, New York, 2003a.

May M. Nerve Substitution Techniques: XII-VII Hook-Up. XII-VII Jump Graft, and Cross-Face Graft. In Facial Paralysis, Rehabilitation Techniques, pp 61-83. Eds May M, Schaitkin B. Thieme, New York, 2003b.

May M. Muscle Transposition Techniques: Temporalis, Masseter, Digastric. In Facial Paralysis, Rehabilitation Techniques, pp 85-115. Eds May M, Schaitkin B. Thieme, New York, 2003c.

Mayou BJ, Watson JS, Harrison DH, Parry CBW. Free microvascular and microneural transfer of the extensor digitorum brevis muscle for the treatment of unilateral facial palsy. Br J Plast Surg 34:362-67, 1981.

Mc Laughlin CR. Surgical support in permanent facial paralysis. Plast Reconstr Surg 11: 302, 1953.

Millesi H, Meissl G, Berger A. The interfascicular nerve-grafting of the median and ulnar nerves. J Bone Joint Surg 54: 727-50, 1972.

Millesi H. Nerve grafting. In Microreconstruction of Nerve Injuries, pp 223-27. Ed. Terzis JK. WB Saunders, Philapdelphia 1987.

Millesi H. Indications and technique for nerve grafting. In Operative Nerve Repair and reconstruction, pp 525-543. Ed Gelberman R., JB Lippincott, Philadelphia 1991.

Murphy WA, Totty WG, Carroll JE. MRI of normal and pathologic skeletal muscle. Am J Radiol 146: 565-74, 1986.

Murty GE, Diver JP, Kelly PJ, O'Donoghue GM, Bradley PJ. The Nottingham system: objective assessment of facial nerve function in the clinic. Otolaryngol Head Neck Surg, 110: 156-61, 1994.

Myckatyn TM, Mackinnon SE. A review of facial nerve anatomy. Semin Plast Surg 18; 1: 5-11, 2004.

Nehrer-Tairych G, Rab M, Kamolz L, Detinger M, Stöhr H, Frey M. The influence of the donor nerve on the function and morphology of a mimic muscle after cross innervation: an experimental study in rabbits. Brit J Plast Surg 53: 669-75, 2000.

Novak C. Rehabilitation strategies for facial nerve injuries. Semin Plast Surg 18: 47-51, 2004.

O´Brien B, Franklin JD, Morrison WA. Cross-facial nerve grafts and microneurovascular free muscle transfer for long established facial palsy. Brit J Plast Surg 33: 202-15, 1980.

O`Brien BMcC, Pederson WC, Khazanchi RK, Morrison WA, MacLeod AM, KumarV. Results of management of facial palsy with microvascular free-muscle transfer. Plast Reconstr Surg 86:12-22, 1990.

Oldfors A, Mair WGP, Fogdestam I. The morphological sequence in man of de- and reinnervation in free muscle tranfer with microneurovascular anastomoses. Scand J Plast Reconstr Surg 23: 35-42, 1989.

Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D et al. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet 28: 131-38, 2001.

Oudega M, Hagg T. Nerve growth factor promotes regeneration of sensory axons into adult rat spinal cord. Exp Neurol 140: 218-29, 1996.

Paletz JL, Manktelow RT, Chaban R. The shape of a normal smile: implications for facial paralysis reconstruction. Plast Reconstr Surg 93: 784-89, 1994.

Rab M, Haslik W, Grunbeck M, Schmidt M, Gradl B, Giovanoli P, Frey M. Free functional muscle transplantation for facial reanimation: experimental comparison between the one- and two-stage approach. JPRAS 59: 797-806, 2006.

Rantanen J, Ranne J, Hurme T, Kalimo H. Denervated segments of injured skeletal muscle fibers are reinnervated by newly formed neuromuscular junctions. J Neuropathol Exp Neurol 54: 188-94, 1995.

Rende M, Giambanco I, Buratta M, Tonali P. Axotomy induces a different modulation of low-affinity nerve growth factor receptor and choline acetyltransferase between adult rat spinal and brainstem motoneurons. J Comp Neurol 363: 249-63, 1995.

Rovak JM, Tung TH, Mackinnon SE. The surgical management of facial nerve injury. Semin Plast Surg 18(1): 23-29, 2004a.

Rovak JM, Mungara AK, Mustafa AA, Cederna PS. Effects of Vascular Endothelial Growth Factor on nerve regeneration in acellular nerve grafts. J Reconstr Microsurg 20 (1): 53-58, 2004b.

Rowlands S, Hooper R, Hughes R, Burney P. The epidemiology and treatment of Bell's palsy in the UK**.** Eur J Neurol 9(1):63-7, 2002

Roy RR, Pierotti DJ, Flores V, Rudolph W, Edgerton VR. Fibre size and type adaptions to spinal isolation and cyclical passive stretch in cat hindlimb. J Anat 180: 491-99, 1992.

Rubin L. Re-animation of the Paralyzed Face. St Louis, CV Mosby, 1977.

Rudkin GH, Miller TA. Repair, Regeneration and Grafting of Skeletal Muscle. In Plastic Surg. pp 605-619. Ed Mathes SJ. Saunders, Philadelphia, 2006.

Russo CP, Smoker WRK, Weissman JL. MR appearance of trigeminal and hypoglossal motor denervation. AJNR Am J Neuroradiol 18: 1375-83, 1997.

Saika T, Senba E, Noguchi K et al. Effects of nerve crush and transsection on mRNA levels for nerve growth factor receptor in the facial motoneurons. Mol Brain Res 9: 157-60, 1991.

Salinas RA, Alvarez G, Ferreira J. Corticosteroids for Bell's palsy (idiopathic facial paralysis). Cochrane Database Syst Rev 1, 2008.

Salmi A, Lamminen A, Tukiainen E, Asko-Seljavaara S. Magnetic resonance imaging of free muscle flaps. Eur J Plast Surg 19: 21-25, 1995.

Sargent EW, Fadhli OA, Cohen RS. Measurement of facial movement with computer software. Arch Otolaryngol Head Neck Surg 124: 313-18, 1998.

Sassoon EM, Poole MD, Rushworth G. Reanimation for facial palsy using gracilis muscle grafts. Brit J Plast Surg 44: 195-200, 1991.

Scaramella LF, Tobias E. Facial nerve anastomosis. Laryngoscope 83: 1834-40, 1973.

Schmalbruch H, Al-Amood WS, Lewis DM. Morphology of long-term denervated rat soleus muscle and the effect of chronic electrical stimulation. J Physiol 441: 233-41, 1991.

Schmalbruch H, Lewis, DM. A comparison of the morphology of denervated with aneurally regenerated soleus muscle of rat. J Muscle Res Cell Motil 15: 256-66, 1994.

Schmidt CE, Leach JB. Neural Tissue Engineering: Strategies for Repair and Regeneration. Ann Rev Biomed Eng Vol 5: 293- 348, 2003.

Schor N. The p75 neurotrophin receptor in human development and disease. Prog Neurobiol 77: 201-14, 2005.

Schultes G, Gaggl A, Kleinert R, Kärcher H. Vascularized vs. non-vascularized nerve transfers: histologic study in rats. J Reconstr Microsurg 17 (8): 637-42, 2001.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219: 983-85, 1983.

Sesodia S, Cullen MJ. The effect of denervation on the morphology of regenerating rat soleus muscles. Acta Neuropathol 82(1): 21-32, 1991.

Shenaq SM, Kim JY. Repair and Grafting of Peripheral Nerve. In Plastic Surgery, Vol I, pp720-22. Ed Mathes SJ. Saunders, Philadephia 2006.

Shindo M. Management of facial paralysis. Otolaryngol Clin North Am 32: 945-64, 1999.

Simovic D, Isner JM, Ropper AH, Pierczek A, Weinberg DH.Improvement in chronic ischemic neuropathy after intramuscular phVEGF165 gene tranfer in patients with critical limb ischemia. Arch Neurol 58: 761-68, 2001.

Slater CR, Schiaffino S. Innervation of regenerating muscle. In Skeletal Muscle Repair and Regeneration, pp.303-33. Eds Schiaffino S, Partridge T. Springer Science and Business Media, Netherlands, 2008.

Smith, JW. A new technique of facial animation. In Transactions of the 5th International Congress of Plastic Surgery, p83-84.Ed Hueston JH. Butterworths, London, 1971.

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-spesific receptor for vascular endothelial growth factor. Cell 92: 735-45, 1998.

Sondell M, Lundborg G, Kanje M. Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci 19(14): 5731-40, 1999a.

Sondell M, Lundborg G, Kanje M. Vascular endothelial growth factor stimulates Schwann cell invasion and neovascularization of acellular nerve grafts. Brain Res 846: 219-28, 1999b.

Sondell M, Sundler F, Kanje M. Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. Eur J Neurosci 12:4243-54, 2000.

Squinto S, Stitt T, Aldrich T, Davis S, Bianco S et al. TrkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3, but not nerve growth factor. Cell 65: 885-93, 1991.

Storkebaum E, Lambrechts D, Carmeliet P. VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. Bio Essays 26: 943-54, 2004.

Swartz WM. Free muscle transfers for facial paralysis. In Facial Paralysis, Rehabilitation Techniques, pp.117-26. Eds May M, Schaitkin B. Thieme, New York, 2003.

Takushima A, Harii K, Asato H, Yamada A. Neurovascular free-muscle transfer to treat facial paralysis associated with hemifacial microsomia. Plast Reconstr Surg 109: 1219-27, 2002.

Tate J, Tollefson T. Advances in facial reanimation. Curr Opin Otollaryngol Head Neck Surg 14: 242-48, 2006.

Terzis JK, Sweet RC, Dykes RW, Williams HB. Recovery of function in free muscle transplants using microneurovascular anastomoses. J Hand Surg Am 3: 37-59, 1978.

Terzis JK. Pectoralis minor: a unique muscle for correction of facial palsy. Plast Reconstr Surg 83(5): 767-76, 1989.

Terzis JK: “Babysitters”. An exciting new concept in facial reanimation.In Proceedings of the 6th International Symposium on the Facial Nerve. Rio de Janeiro, Brazil. Kugler and Ghendini Publications, 1990.

Terzis JK, Sun DD, Thanos PK. Historical and basic science review: past, present, and future of nerve repair. J Reconstr Microsurg 13: 215-25, 1997.

Terzis J, Noah M. Analysis of 100 Cases of Free-Muscle Transplantation for Facial Paralysis. Plast Reconstr Surg 99: 1905-21, 1997.

Terzis J, Noah EM. Möbius and Möbius-like patients: etiology, diagnosis and treament options. Clin Plast Surg, 29: 497-518, 2002.

Terzis J. Reanimation of the eye. Lecture in the Facial Palsy Vesalius Course. Brussels, Belgium, 23.-25.9.2004.

Thanos PK, Terzis JK. A histomorphometric analysis of the cross-facial nerve graft in the treatment of facial paralysis. J Rec Microsurg 12(6): 375-82, 1996.

Tuszynski MH, Gabriel K, Gage FH, Suhr S, Meyer S, Rosetti A. Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor, and noradrenergic neurites after adult spinal cord injury. Exp Neurol 137: 157-73, 1996.

Ueda K, Harii K, Asato H, Yamada A. Neurovascular free muscle transfer combined with cross-face nerve grafting for the treatment of facial paralysis in children. Plast Reconstr Surg 101: 1765-73, 1998.

Uetani M, Hayashi K, Matsunaga N, Imamura K, Ito N. Denervated skeletal muscle: MR imaging. Work in progress. Radiology 189: 511-15, 1993.

Vanstapel MJ, Gatter KC, Wolf-Peeters C. New sites of human S-100 immunoreactivity detected with monoclonal antibodies. Am J Clin Pathol 85:160-68, 1986.

Vedung S, Olsson Y. Light- and electron microscopic findings in the distal end of human cross-face sural nerve grafts. Scand J Plast Reconstr Surg 16: 275-81, 1982.

Weis J, Schröder M: Diffrential effects of nerve, muscle and fat tissue on regenerating nerve fibers in vivo. MuscleNerve, 12: 723-34, 1989.

Wester DJ, Whiteman MLH, Singer S, Bowen BC, Goodwin WJ. Imaging of the postoperative neck with emphasis on surgical flaps and their complications. Am J Radiol 164: 989-93, 1995.

Wichester PK, Gonyea WJ. A quantitative study of satellite cells and myonuclei in stretched avian slow tonic muscle. Anat Rec 232: 369-77, 1992.

Von Boguslawsky K. Immunohistochemical detection of progesterone receptors in paraffin sections. A novel method using microwaveoven pretreatment. APMIS 102: 641-46, 1994.

Whitney TW, Buncke HJ, Albert BS, Buncke GM, Lineaweaver WC. The serratus anterior free muscle flaps: experience with 100 concecutive cases. Plast Reconstr Surg 86: 481, 1990.

Wu WT, Chai H, Zhang JY, Gu HY, Xie YY, Zhou LH. Delayed implantation of a peripheral nerve graft reduces motoneuron survival but does not affect regeneration following spinal root avulsion in adult rats. J Neurotraum 21(8): 1050-58, 2004.

Yoshimura K, Harii K, Asato H, Ueda K, Yamada A. Immunohistochemical analysis of Clinically Transplanted Muscles. J Surg Res 79: 31-38, 1998.

Yoshimura K, Harii K. A regenerative change during muscle adaption to denervation in rats. J Surg Res 81: 139-46, 1999.

Yoshimura K, DeMeester J, Hasan JS, Urbanchek MG, Kuzon WM Jr. Neurovascular transfer does not cause skeletal muscle fiber degeneration. Plast Reconstr Surg 117: 1455-61, 2006.

Zhang F, Lineaweaver C, Ustuner T, Kao S, Tonken H, Campagna-Pinto D, Buncke H. Comparison of muscle mass preservation in denervated muscle and transplanted muscle flaps after motor and sensory reinnervation and neurotization. Plast Reconstr Surg 99(3): 803-14, 1997.

Zhang H, Vutskits L, Pepper MS, Kiss JZ. VEGF is a chemoattractant for FGF-2-stimulated neural progenitors. J Cell Biol 163: 1375-84, 2003.

Zuker RM, Eppley BL. Facial reanimation. In Plastic Surgery, Indication, Operation and Outcome, Vol III. Pp 1611-23. Mosby, 2000.

Zuker RM, Goldberg CS, Manktelow RT. Facial animation in children with moebius syndrome after segmental gracilis muscle transplant. Plast Reconstr Surg 106(1): 1-8, 2000.

Zuker R, Manktelow R, Hussain G. Facial paralysis. In Plastic Surgery, Vol III, pp 883-916. Ed Mathes SJ. Saunders, Philadelphia, 2006.

Äärimaa Ville. Regeneration of skeletal muscle after injury- with emphasis on surgical treatment. Turun yliopiston julkaisuja, sarja D, osa 691. Painosalama, Turku, 2006.