

## Histomorphometric Analysis of Nerve Biopsies in the Two-Stage Cross-Face Free Muscle Transfer for Facial Reanimation with Correlation to the Functional Outcome

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### ABSTRACT

Free micro-neurovascular muscle transfer with cross-face nerve graft connected to the contra-lateral intact facial nerve offers the best prospect in restoring muscle tone and voluntary and emotional expressions in long established facial palsy [1].

Histological analysis of nerves involved in the two-stage free muscle transfer for facial reanimation using cross-face nerve graft and free muscle transfer is important to provide an idea about histomorphometric changes that happen before muscle transfer and provide possible explanation to the variation of the clinical results.

Nerve biopsies were taken from 10 patients with unilateral long standing facial paralysis in the period from January 2008 to May 2010 in Liverpool region, north west of England. Age ranged from 7-49 years old. Nerve regeneration was assessed by light and electron microscopes counting numbers of myelinated and un-myelinated axons and measuring their diameters. These neurohistological findings were compared with the functional outcome after completing the surgical procedure.

The study showed characteristic for regeneration in the form of obvious loss of myelinated nerve fibres at the end of the cross-face nerve graft and the presence of a large number of un-myelinated axons that were grouped in clusters. We found a positive correlation between the number and diameter of the regenerating nerve fibres in one hand and the functional outcome in the other hand. There was an inversely proportional relationship between patient's age and the number and diameter of the regenerating nerve fibres.

The established methodology in the present study can be used in future studies with larger numbers of patients to get a statistically significant results.

### INTRODUCTION

Transplantation of functioning muscle to the paralysed face based on microvascular and neural repairs has been accepted as an excellent option for facial reanimation [1]. Successful transplantation achieves closest symmetry at rest as well as voluntary motion and expression in the previously paralysed sagging face. This procedure is usually

done in two stages involving a cross-face nerve graft in the first stage and free muscle transfer in second stage that is done 6-12 months later. This two-staged operation allows a unique chance to study nerve regeneration across the nerve graft in the second stage before being connected to a target organ. Nerve regeneration was assessed by light and electron microscopes measuring the diameters and counting numbers of myelinated and unmyelinated axons. These neurohistological findings were compared with the functional outcome after completing the surgical procedure. The clinical results were assessed by one surgeon, who was not aware of the histology results, using the standard grading system used in an earlier study [2].

We aimed at improving the results of facial reanimation using the two-stage free muscle transfer. This could be achieved if we found a relationship between the histomorphometric findings of the regenerating nerves and the ultimate functional outcome. Some cases in the series of cases mentioned in a previous study [2] had poor results with no obvious reason. We aim at possible prediction of the functional outcome based on the histomorphometric findings. This can guide us to improve the result possibly through change of technique.

*An answer to the following questions was looked for:*

- 1- Is it possible to predict the functional outcome of the free muscle flap based on the results of the histomorphometric analysis of nerve regeneration?
- 2- Does the nerve regeneration and functional outcome depend on the time interval between the two stages of the free muscle transfer?
- 3- Is there any correlation between patient's age and efficiency of nerve regeneration?

## PATIENTS AND METHODS

Nerve biopsies were taken from 10 patients with unilateral long standing facial paralysis undergoing the two stage free muscle transfer in the period from January 2008 to May 2010 in Liverpool region, north west England, UK.

All patients signed an informed consent.

### *Sites for nerve biopsies:*

Nerve biopsies (3.5mm long) were taken from 10 patients with unilateral longstanding facial paralysis who were undergoing the two-stage free gracilis transfer (cross-face nerve graft in the first stage and free muscle transfer in the second stage).

*In the first stage, biopsy specimens were taken from:*

- The normal facial nerve branches selected to re-innervate the muscle transplant on the normal side of the face.
- The distal end of sural nerve graft.

*In the second stage nerve biopsies were taken from:*

- The distal end of the cross-face nerve graft.
- The end of the nerve to gracilis (Fig. 1).

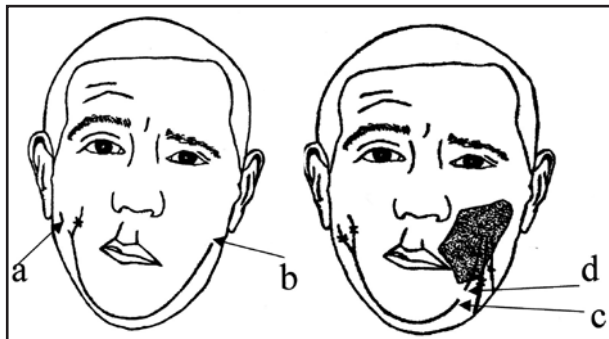


Fig. (1): Sites of nerve biopsy in the two-stage free gracilis transfer: a) From normal facial nerve in the 1<sup>st</sup> stage b) From distal end of sural nerve graft in the 1<sup>st</sup> stage c) From distal end of cross-face nerve graft in the 2<sup>nd</sup> stage (after a mean of 12 months) d) From distal end of gracilis nerve.

All the 10 patients completed the two stages giving full set of biopsies.

Nerve specimens were preserved in 10% formaline and were sent in the same day to the neuropathology laboratory at the Walton neuro-centre in Fazakerley, Liverpool, UK.

### *Histotechnical methods:*

Preparation of nerve specimens for light and electron microscopy was performed by neuropathology technicians at the Walton neuro-centre as follows:

After 24 hours formaline fixation, nerves were trimmed into fascicles or group of fascicles of maximum 2mm diameter and 3-4mm long. Nerves were washed in Cacodylate buffer (PH 7) then processed into super resin according to the following schedule:

- One hour 1% Osmium Tetroxide
- Five minutes wash into 50% Alcohol
- Fifteen minutes in 1% Uranium Acetate in 50% Ethanol
- Fifteen minutes in 50% Ethanol
- Fifteen minutes in 70% Ethanol
- Fifteen minutes in 90% Ethanol
- Fifteen minutes in absolute Ethanol (three changes)
- Thirty six hours in Super Resin

Then the fascicles were polymerised at 60° C overnight using flat embedding trays so that nerve can be orientated. An ultramicrotome and disposable glass knives were used to cut 0.6 mm sections of the nerve specimen. Then sections were dried at 80° in hot plate for at least 2 hours. Staining of nerves was done using 0.5% Toluidine blue in 1% borax in the hot plate for 30 seconds. Then sections were washed in tap water and allowed to dry on a hot plate for at least 2 hours before mounting. The fascicles to be viewed by electron microscope (EM) were selected and cut into ultra thin sections (Gold colour of section indicates 60-90 nm thick). Then sections were now laid on a nickel grid to be ready for EM examination (3).

A qualitative and quantitative histomorphometric study was performed on all nerve specimens using both light microscopy (Leica) and electron microscopy (Phillips TEM 400) and photographs were taken.

Light microscopy was used to count and measure the diameter of the myelinated nerve fibres as well as looking for any abnormal cellular findings that might be relevant to the results. The magnification used was 40X10. A specially designed computer program was used to count myelinated nerve fibres and measure their diameters. Electron microscopy was used to count and measure the diameter of the unmyelinated nerve fibres as well as estimating the percentage of collagen fibres in the nerve specimen.

One surgeon independently of the results of nerve regeneration assessed the clinical results of

the patient using a standard facial grading system formed of seven assessment criteria that was used in an earlier study [2] (Table 1).

All specimens were examined by the same neuropathologist who was unaware of the clinical results.

Table (1): Standard criteria for assessment of operative results (Grading: 1= Poor, 2= Fair, 3= Good)

No	Criteria
I	<i>Symmetry of mid face at rest:</i> 1- Marked asymmetry; midline pull to other side by normal facial muscles 2- Mild asymmetry of oral commissure 3- No detectable asymmetry
II	<i>Symmetry of mid face on smiling:</i> 1- Marked asymmetry; midline pull to other side by normal facial muscles 2- Mild asymmetry 3- No obvious asymmetry
III	<i>Muscle bulk:</i> 1- Marked cheek bulk producing asymmetry 2- Mild increase in cheek bulk 3- No obvious increase in cheek bulk
IV	<i>Independent movement of the transferred muscle:</i> 1- No independent contraction seen 2- Partial contraction of muscle requires strong contraction of the normal side 3- Good independent muscle contraction with no movement of the normal side
V	<i>Elevation of nasolabial fold:</i> 1- No elevation 2- Mild elevation 3- Normal elevation with good facial symmetry
VI	<i>Ability to close the mouth:</i> 1- Oral incompetence 2- Weak incomplete mouth closure 3- Normal mouth closure
VII	<i>Involuntary movements of the transferred muscle:</i> 1- Marked involuntary movement with dyskinesia or mass movement of the face 2- Mild involuntary movement; only with strong contraction of the other side 3- No noticeable involuntary movement

## RESULTS

### *Patients criteria:* (Tables 2,3)

The mean age was 35.9 years old with (Range 7-49 years old). The mean interval for nerve regeneration between 1<sup>st</sup> and 2<sup>nd</sup> stages of the operation was 11.5 months (Range 10-13 months).

### *The light microscopic findings:* (Table 4)

In cross-face nerve graft specimens, cross sections showed preservation of the fascicular pattern with a surrounding thickened perineurium. The epineurium appeared normal. There were numerous endoneurial fibroblasts and Schwann cells but rather few myelinated nerve fibres.

The other nerves (sural, nerve to gracilis and facial) contained a large number of normal myelinated fibres that showed myelin vacuulations of varying degree in most specimens. Few groups of un-myelinated nerve fibres were also found.

The mean number of myelinated nerve fibres in the normal sural nerve was 4063 per mm<sup>2</sup> (Range 3047-4805). It was 3906 per mm<sup>2</sup> (Range 3398-4453) for the normal facial nerve, 3646 per mm<sup>2</sup> (Range 3477-3945) for the normal nerve to gracilis and it was 182 per mm<sup>2</sup> (Range 78-273) for the distal end of the cross-face nerve graft.

The mean of minor diameters of the myelinated nerve fibres for the normal sural nerve in all cases was 10.4 µm (Range 5.12-20.27). It was 6.37 µm (Range 4.89-8.42) for the normal facial nerve, 7.86 µm (Range 7.16-8.63) for the normal nerve to gracilis and 4.08 µm (Range 2.15-8.05) for the distal end of the cross-face nerve graft. Figures II to V show the light microscopic pictures of normal sural, cross-face nerve graft after 10 months of regeneration, normal nerve to gracilis and normal donor facial nerve respectively.

### *The electron microscopic findings:* (Table 5)

The electron microscopic findings confirmed observations from the light microscopy and added some further information.

In all cases of the cross-face nerve graft specimens, the endoneurium showed numerous axons of which few were fully myelinated. The un-myelinated nerve fibres appeared in the form of regenerating clusters. Reich granules (inclusion bodies) were seen in some of the specimens.

The mean number of un-myelinated nerve fibres calculated from the normal sural nerve was 36374 per mm<sup>2</sup> (Range 23142-62524). It was 46629 per mm<sup>2</sup> (Range 32074-71050) for the normal facial nerve, 41460 per mm<sup>2</sup> (Range 31000-52780) for the normal nerve to gracilis and it was 100990 per mm<sup>2</sup> (Range 53592-178640) for the distal end of the cross-face nerve graft.

The mean of minor diameters of the unmyelinated nerve fibres for the normal sural nerve in all cases was 0.78  $\mu\text{m}$  (Range 0.58-0.89). It was 1.01  $\mu\text{m}$  (Range 0.84-1.26) for the normal facial nerve, 0.85  $\mu\text{m}$  (Range 0.75-0.94) for the normal nerve to gracilis and 0.77  $\mu\text{m}$  (Range 0.45-1.19) for the distal end of the cross-face nerve graft. Figs. (6-9) show the electron microscopic pictures of normal sural, cross-face nerve graft after 10 months of regeneration, normal nerve to gracilis and normal donor facial nerve same patient respectively.

All cases showed significant increase in endoneurial collagen and a significant amount of oedema.

Table (2): Patients criteria.

Criteria	Two-stage (n = 10)
Age, years [Mean (SD)]	26.62 (15.36) Range (7-49 years)
Adult: Child	7:3
Gender (♂: ♀ ratio)	1:9
Duration between 1 <sup>st</sup> & 2 <sup>nd</sup> Stages, months [Mean (SD)]	10.13 (5.07) Range (10-13 months)

#### Clinical results: (Table 6)

All patients had completed the two-stage gracilis transfer and had their operative results assessed. Using the standard grading system and the points system mentioned earlier (Table 1), each patient had a score out of 21 which is the sum of the grades of the seven criteria. Two patients had bad overall results due to muscle graft failure (had a score of <14/21). Three patients had fair overall results with score of 14-16/21. Five patients had good overall results with excellent muscle excursion on smiling and scored >16/21.

Table (3): Aetiology of facial palsy.

Etiology	No
Congenital	6
Removal of acoustic neuroma	2
Bell's palsy	1
Parotidectomy	1
Total	10

Table (4): Mean number and diameter of myelinated nerve fibres.

	Sural nerve	Facial nerve	Cross-face nerve graft	Nerve to gracilis
Mean number (range) per $\text{mm}^2$	4063 (3047-4805)	3906 (3398-4453)	920 (78-3135)	3646 (3477-3945)
Mean of minor diameters (range) $\mu\text{m}$	10.4 (5.12-20.27)	6.37 (4.89-8.42)	4.08 (2.15-8.05)	7.86 (7.16-8.63)

Table (5): Mean number and diameter of myelinated nerve fibres.

	Sural nerve	Facial nerve	Cross-face nerve graft	Gracilis nerve
Mean number (range) per $\text{mm}^2$	36374 (23142-62524)	46629 (32074-71050)	100990 (53592-178640)	41460 (31000-52780)
Mean of minor diameters (range) $\mu\text{m}$	0.78 (0.58-0.89)	1.01 (0.84-1.26)	0.77 (0.54-1.19)	0.85 (0.75-0.94)

Table (6): The overall examination results.

Grade	Number of patients
Good >16	5
Fair 16-14	3
Poor <14	2

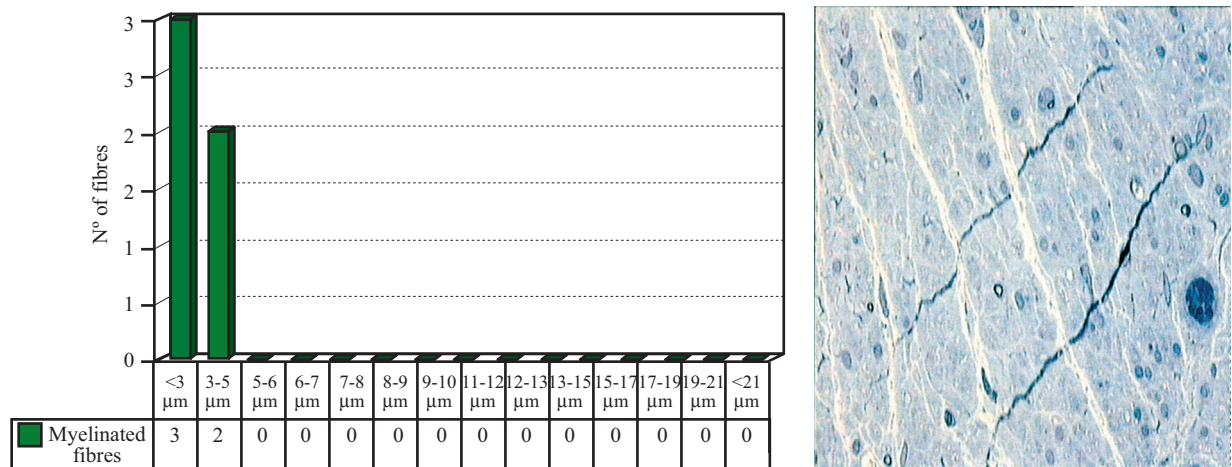


Fig. (2): Semi-thin resin section of normal sural nerve (right). Note the proximate density of myelinated fibers. A representing chart (left).

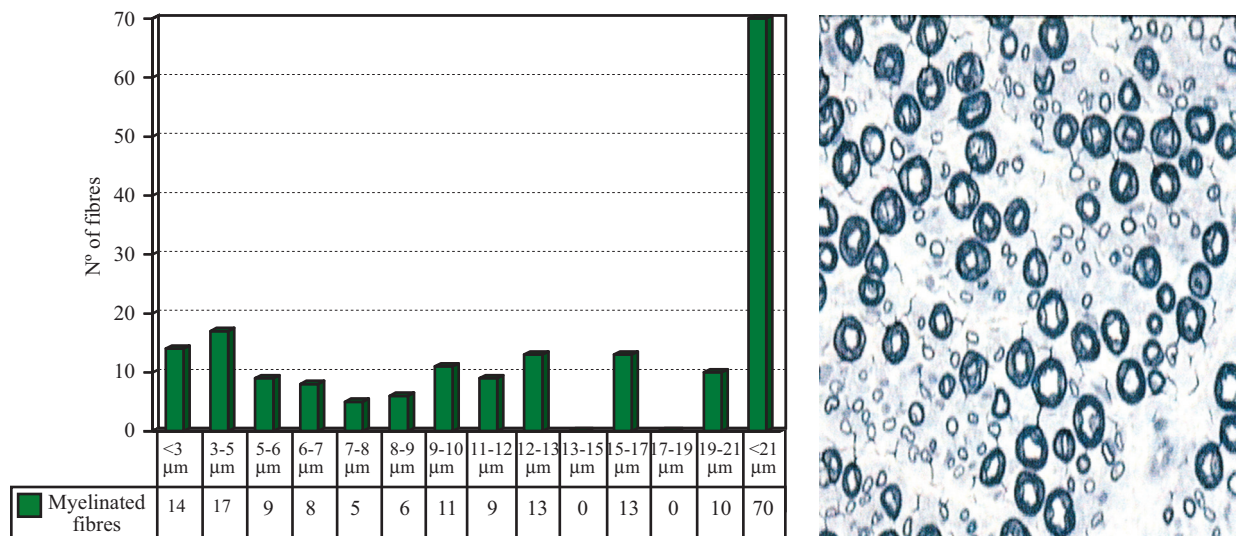


Fig. (3): Semi-thin resin section of cross face nerve graft after 10 months of regeneration. Note scanty myelinated fibers compared to the above figure. A representing chart (left).

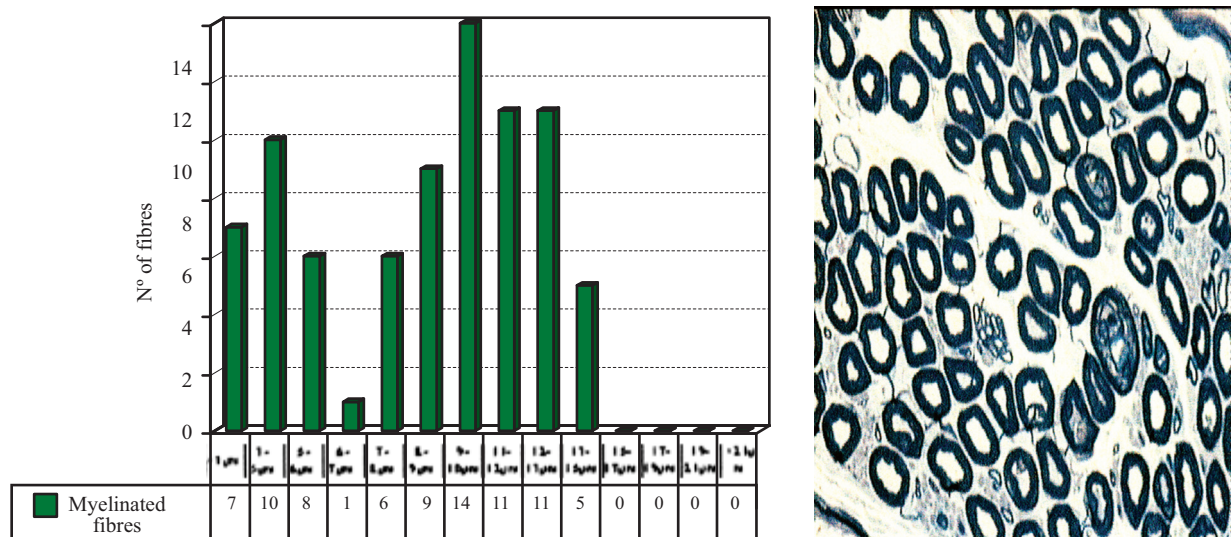


Fig. (4): Semi-thin resin section of normal gracilis nerve (right). A representing chart (left).

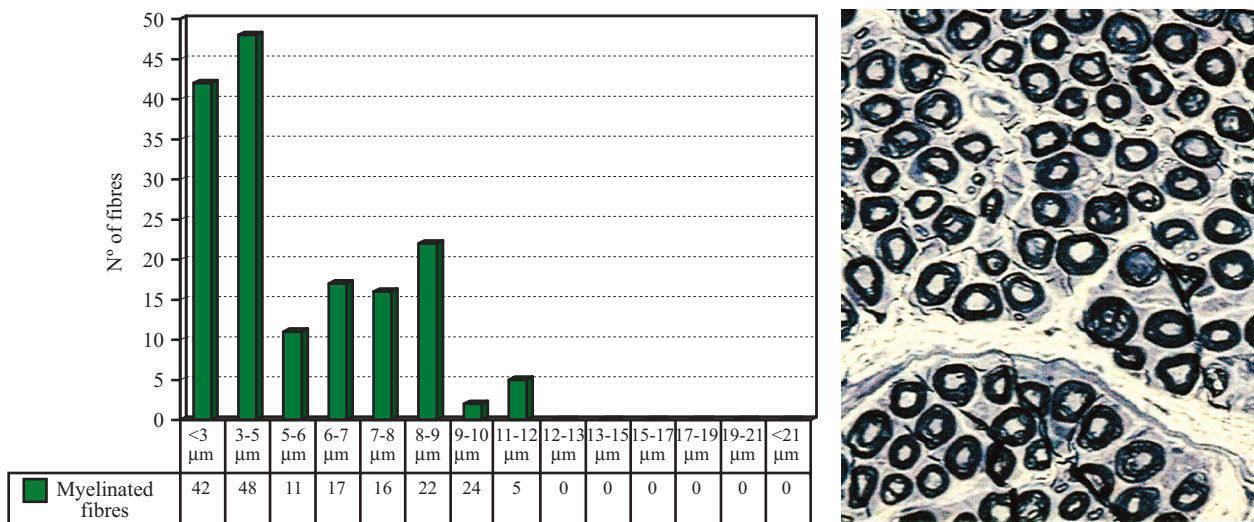


Fig. (5): Semi-thin resin section of normal facial nerve (right). A representing chart (left).

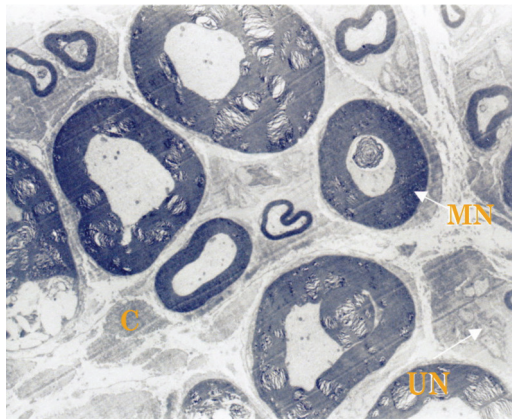


Fig. (6): Electron micrograph of normal sural nerve taken in the first stage. Note the heavily myelinated normal nerve fibers with minimal amount of collagen compared to figure VII (MN = myelinated normal nerve fibers; UN = unmyelinated nerve fibers; C = collagen).

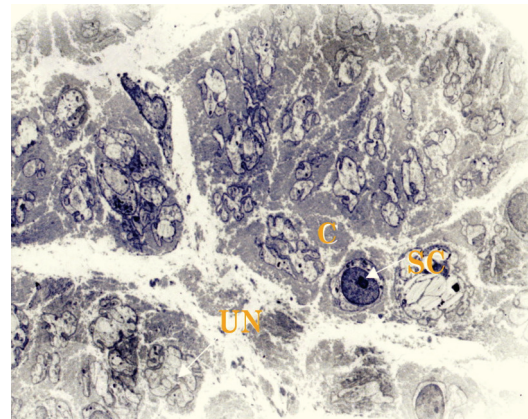


Fig. (7): Electron micrograph of cross-face sural nerve graft taken in the second stage of the same patient after 10 months. Numerous unmyelinated nerve fibers and no myelinated nerve fibers are seen. Markedly increase in the amount of collagen (MN = myelinated normal nerve fibers; UN = unmyelinated nerve fibers; C = collagen; SC = Schwann cell).

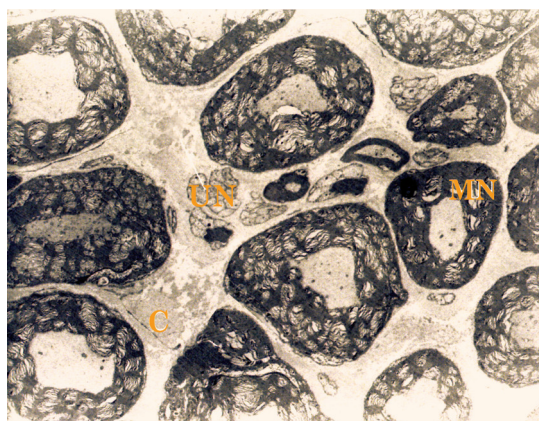


Fig. (8): Electron micrograph of normal gracilis nerve of the same patient taken in the second stage. Note few unmyelinated nerve fibers with minimal amount of collagen. Myelinated nerve fibers show myelin vacuolation. (MN = myelinated normal nerve fibers; UN = unmyelinated nerve fibers; C = collagen).

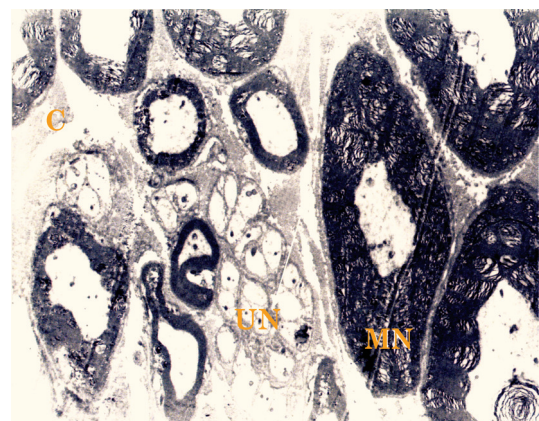


Fig. (9): Electron micrograph of normal donor facial nerve of the same patient taken in the first stage. Note few big sized unmyelinated nerve fibers with minimal amount of collagen (MN = myelinated normal nerve fibers; UN = unmyelinated nerve fibers; C = collagen).

## DISCUSSION

Histological analysis of nerves involved in the two-stage free muscle transfer for facial reanimation using cross-face nerve graft and free muscle transfer is important to provide an idea about histomorphometric changes that happen before muscle transfer and provide possible explanation to the variation of the clinical results. The two staged operation allows an opportunity to take a biopsy from the distal end of the cross-face nerve graft inserted in the first stage after about ten months interval prior to muscle grafting.

A similar study by Frey et al. [4] examined the cross-face nerve grafts from seven patients. They used the gracilis muscle as the donor motor muscle. After a regeneration period of 10-14 months, there was a total loss of large myelinated nerve fibres with a reduced amount of small myelinated nerve fibres (32-251/mm<sup>2</sup>). They found no relation between the total number and the diameter of regenerated myelinated nerve fibres in the distal end of the nerve graft and the functional results [4]. From findings in the present study, we noticed a positive correlation between the number and diameter of the regenerating nerve fibres in one hand and the functional outcome in the other hand in contrast to Frey's findings. However the relatively small number of cases in either studies did not allow establishing a statistically significant correlation between clinical results and the histological findings. The established methodology in the present study can be used in future studies with larger numbers of patients to get significant results.

Such a low number of nerve fibres found at the distal end of cross-face nerve graft in the second stage of the operation was surprising to the neuropathologists looking after this research project and their explanation was because the nerve was not connected to a target organ (a muscle).

In the present study, electron microscopic examination showed some interesting findings. The presence of the unmyelinated fibres in groups at the end of the cross-face nerve graft (Fig. 7). These groups are usually referred to as regenerating clusters by neuropathologists. These regenerating clusters are markers for axonal regeneration [5]. Myelin vacuulations found in some of the nerve specimens are probably due to inadequate preservation or poor surgical handling leading to artefacts (Fig. 8). Reich granules seen in (Fig. 7) are just inclusion bodies usually encountered in the regenerating nerves. Their presence does not mean any abnormal pathology.

In the present study, the normal donor facial nerve branch was found to have a mean of 3906/mm<sup>2</sup> myelinated nerve fibres with a mean diameter of 6.37  $\mu$ m for each fibre. Frey et al., found that the mean number of myelinated nerve fibres in the facial nerve was 834/mm<sup>2</sup> with a mean diameter of 9.25  $\mu$ m. The big variation in numbers in both studies is probably due to the variation in the size of the donor facial nerve branch which was the buccal in most cases in the present study. Frey did not define the facial nerve branch used in his cases but he advised utilising facial nerve branch other than the buccal branch to get a functionally faster motor nerve to the transferred muscle [4].

In the present study, the mean number of myelinated nerve fibres in the distal end of normal sural nerve graft was 4063 per mm<sup>2</sup> with a mean diameter of 10.4  $\mu$ m for each fibre. In Frey's study [6], the mean number of myelinated nerve fibres in the normal sural nerve was 1074 per mm<sup>2</sup> with a mean diameter of 5.68  $\mu$ m for each fibre. In a study by Dyck et al., the distal sural nerve was found to have 8800 myelinated nerve fibres per mm<sup>2</sup> [7]. No explanation to these variations was found.

In a study by Vedung et al., they performed light and electron microscopic study on the distal end of cross-face nerve graft before free muscle transplantation in five patients [8]. A very large number of unmyelinated axons and few myelinated axons were found early in the regeneration process (4-6 months). A considerable fraction of myelinated axons were found particularly in fascicles removed 12-13 months after the nerve operation. A marked increase in endoneurial collagen was found and at the very tip, a neuroma was present. They stated that these findings were important prerequisites for successful future neurotization of the transplanted muscle. They did not evaluate the clinical results, as the follow-up period was too short. They did not quantify their results that made it difficult to hold a comparison between their results and the present study. In the present study, no neuromas were seen at the distal end of the cross-face nerve graft.

Electron microscopic studies of distal end of the cross-face nerve graft by Jacobs et al. [9] and Vedung et al. [8] showed a huge number of unmyelinated nerve fibres and suggested that these nerve fibres were capable of transforming into myelinated nerve fibres after connection to a target organ (the donor muscle). In the present study, the electron microscopic findings confirmed the above mentioned findings.

In a study by Frey et al. [6], they advised to perform the second stage free gracilis transfer

operation without delay once the Tinel's sign reached the end of the graft. This is because they found that with increasing regeneration time, the number of myelinated fibres decreased in the contralateral grafts. In the study by Jacobs et al., they stated that there was no effect of delay upon the eventual functional outcome [9]. In the present study, no such correlation could be established due to limited variation in the duration between the first and the second stages in the limited number of patients involved in the research.

In the same previous study [9], authors found no correlation between age and the results of nerve regeneration. In another experimental study by Campbell et al. [10], nerve fibre regeneration was found less efficient in older animals [10]. The seven years old child in the present study showed a high number of myelinated nerve fibres in the distal end of the cross-face nerve graft (3135 per mm<sup>2</sup>). This may indicate a correlation between age and quantitative findings in the regenerating nerve. Further studies are required to establish such a correlation.

More recent studies by Ylä-Kotola et. al., [11] found that there is an increased expression of p75 nerve growth factor receptor (p75NGFR) in human cross-face nerve grafts in the two-stage cross-face facial reanimation, especially in younger patients. They suggested that p75NGFR expression might be a contributing factor in a successful axonal regeneration and eventual recovery of muscle function [11]. They also found that the vascular endothelial growth factor (VEGF) and its receptors are expressed in human cross-face nerve grafts and they compared it with basic histology and p75 nerve growth factor receptor expression of the nerve graft and functional outcome of patients [12]. There are valuable immunohistochemical studies that need to be correlated also with the histomorphometric studies mentioned above to get more detailed information about what happens with two stage cross-face nerve graft operative procedure for facial reanimation.

#### *Summary and Conclusions:*

*Observations came out from the present study are:*

- Information about the changes that happen at the distal end of the cross-face nerve graft after a period of regeneration was mentioned. Despite the obvious loss of myelinated nerve fibres at the end of the cross-face nerve graft, there were a large number of un-myelinated axons that were grouped in clusters characteristic for regeneration. Myelinated fibres were probably replaced with such a large amount of collagen noticed in all cross-face nerve graft specimens.

- A positive correlation between the number and diameter of the regenerating nerve fibres in one hand and the functional outcome in the other hand. There was an inversely proportional relationship between patient's age and the number and diameter of the regenerating nerve fibres.
- Another benefit from this study is establishing the methodology for this research project so that it can be continued later on. A larger series of cases could possibly bring more significant results.

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