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Indices of extracellular matrix turnover in human masseter muscles as markers of craniofacial form—a preliminary study

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SUMMARY Environmental remodelling of the craniofacial musculature is obligatory for successful outcomes following interventions such as functional appliance therapy or orthognathic surgery. Genetically driven remodelling of the craniofacial musculature is also seen in individuals with altered facial form. The processes that are involved in the remodelling of intramuscular connective tissue need to be activated in such situations. Such processes require activity of matrix metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs), which are responsible for extracellular matrix (ECM) turnover. The aim of this study was, therefore, to establish the expression of MMP-2 and MMP-9 and their inhibitors, TIMP-1 and TIMP-2, in the masseter muscle of humans with both normal and increased vertical facial form and to assess whether this expression had any value as a predictor of facial form.

Biopsies were taken from 20 subjects (10 with vertical facial deformity and 10 with normal vertical facial form to act as a control group). The sample group consisted of 15 females and 5 males and the average age of the donors ± standard deviation (SD) was 26.04 ± 6.16 years (range: 17.67–31.25 years). Biopsy samples were then subjected to zymography and reverse zymography to assess MMP and TIMP expression, respectively. Lateral skull cephalograms were analysed for each subject using Spearman's rho correlation coefficients and Mann–Whitney *U*-tests.

TIMP-1 activity was consistently expressed in human masseter muscle. MMP-2, MMP-9, and TIMP-2 activity, when detected, was at a low level. These data indicate that in most individuals, an excess of TIMP-1, compared with MMP-2 and MMP-9, limits ECM turnover in human masseter muscle. There was a demonstrable variation in proteinase expression between different individuals. These preliminary findings, however, do not confirm that indices of ECM turnover are a reflection of an individual's vertical facial form.

Introduction

The precise level and mode of interaction between facial form and muscle function have perplexed orthodontists for several decades. Studies have shown that in comparison with individuals with 'normal' or average facial dimensions, subjects with vertical facial deformities possess either strong or weak masticatory muscles depending upon whether they have reduced or increased facial dimensions, respectively (Ingervall and Helkimo, 1978; Proffit *et al.*, 1983).

Muscle function, however, is simply a reflection of its underlying structure. Skeletal jaw muscles are composed of elongated multinucleate muscle fibres encased in connective tissue sheaths. These muscle fibres contain the proteins that are required for muscle contraction. It is perhaps not surprising therefore that subjects with either increased facial dimensions or reduced vertical facial form have been shown to demonstrate a masticatory muscle fibre profile which is at variance from that seen in individuals with a normal vertical facial form (Boyd *et al.*, 1984). What are less well investigated are changes in connective tissue in muscles from such subjects. It is well known that remodelling of the connective tissue of skeletal muscle is the key in many processes; therefore, it follows that remodelling masticatory muscles either through environmental interventions such as functional appliance therapy or through developmental situations, such as seen in the development of abnormal facial form, would require activation of connective tissue remodelling processes.

As has been reviewed extensively in the non-orthodontic literature, remodelling of skeletal muscles in a variety of situations requires the activity of a family of enzymes known as the matrix metalloproteinases (MMPs; Lewis *et al.*, 2001; Carmeli *et al.*, 2004; Anderson, 2006). This family of zinc metalloenzymes is secreted by a wide variety of living cells in tissues and, depending upon the enzyme, acts upon a range of extracellular matrix (ECM) proteins. In the case of skeletal muscle, these proteins are mainly collagen (with some fibronectin) and basement membrane proteins (e.g. laminin). There are multiple levels of control of the activity of these enzymes as either too much or too little activity could have catastrophic effects on tissues. As well as control of the production of enzymes, these tissues can also control the degree of activity of those enzymes.

Finally, tissues also synthesize proteins known as the tissue inhibitors of matrix metalloproteinase (TIMPs), which bind directly to active enzymes to prevent them working (Gomez *et al.*, 1997). The MMPs, MMP-2 and MMP-9 (also known as gelatinases due to the nature of their enzymatic activity), have been the focus of a number of studies, not

least because they degrade the major components of the basal lamina, a barrier that myoblasts must overcome during muscle development and regeneration. It is these two MMPs, MMP-2 and MMP-9, that have been demonstrated in regenerating skeletal muscle (Kherif *et al.*, 1999; Frisdal *et al.*, 2000) and pathological states including muscular dystrophy (Choi and Dalakas, 2000). Previously, MMP and TIMP protein and its location in human craniofacial muscle were identified; the data indicated that TIMP-1 protein expression was predominantly seen with little or no MMP protein (Singh *et al.*, 2000). Although no activity assays were performed, the conclusion was that the predominance of the inhibitors over the enzymes leads to matrix-degrading activity being minimal in normal adult muscle.

The aim of this investigation was to examine the activity of MMP-2 and MMP-9 together with their inhibitors, TIMP-1 and TIMP-2, in subjects with normal and extreme vertical facial development but in whom there was no known systemic muscle pathology. In addition, any association between the expression of these gelatinases, together with their inhibitors, and an individual's facial form was investigated.

Materials and methods

Subjects

A total of 20 subjects were recruited for each part of the study. Due to the destructive nature of the methodology, there was not always a sufficient sample to conduct all the analyses on all the individual patient samples. Consequently, two independent groups of subjects for each set of analyses (MMP and TIMP) were identified. The experimental group comprised 10 subjects who were scheduled to undergo orthognathic surgery for the correction of vertical facial deformity at the University College London Hospital, UK. A further 10 subjects comprised a control group who were receiving surgery for the correction of predominately horizontal discrepancies and in whom the vertical facial form was considered normal (confirmed by cephalometry). Biopsies were taken from the deep surface of the anterior border of the superficial belly of the masseter muscle via an intraoral approach at the time of surgery (Boyd *et al.*, 1984). Removal of biopsy tissue was approved by, and in accordance with, local ethical approval. All 20 subjects in each group provided informed consent. There were 15 female and 5 male subjects (26.04 ± 6.16 years, range: 17.67–31.25 years) with no history of connective tissue disorders, myopathies, endocrine disorders, autoimmune disease, bone disease, bleeding disorders, or regular use of prescribed drugs. Biopsies were transported to the laboratory in sterile, saline-moistened gauze and processed immediately.

Cephalometric analysis

Cephalometric analysis was used to classify the masseter muscle samples according to vertical facial form. The analysis consisted of seven points [sella, nasion, articulare (Ar), anterior nasal spine (ANS), posterior nasal spine (PNS), menton (Me), and gonion (Go)] and three planes (sella to nasion, maxillary, mandibular) identified from lateral cephalograms, of all subjects, taken prior to the start of treatment (Figure 1). The following five parameters were then measured on each lateral cephalogram: SNMnP, the angle formed by the sella–nasion (SN) line and the mandibular plane (MnP); MMPA, the angle formed by the maxillary and mandibular planes; ArGoMe, the gonial angle formed from the points of Ar, Go, and Me; LAFH per cent, the ratio of lower anterior face height (LAFH) to total anterior face height (TAFH) [LAFH was constructed by drawing a perpendicular line from the maxillary plane to Me. Similarly, a line perpendicular from the maxillary plane to nasion represented upper anterior face height (UAFH)]; PFH/TAFH per cent, the ratio of posterior face height (PFH) to TAFH. PFH was constructed by summing the perpendicular distance from sella to the maxillary plane

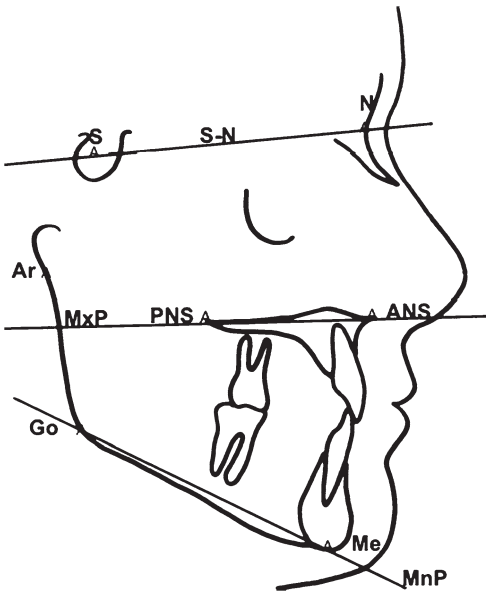

Figure 1 Reference points and planes used in the cephalometric analysis (British Standards Institution, 1983). Points—S, sella: the centre of sella turcica, determined by inspection; N, nasion: the most anterior point of the suture between the frontal and the nasal bones; Ar, articulare: the point of intersection of the dorsal contours of the posterior border of the mandible and the temporal bone; ANS, anterior nasal spine: the tip of ANS as seen on the lateral skull radiograph; PNS, posterior nasal spine: the tip of the posterior spine of the palatine bone in the hard palate; Me, menton: the lowest point on the bony outline of the mandibular symphysis; Go, gonion: the most lateral external point at the junction of the horizontal and ascending rami of the mandible. It is located by bisecting the angle formed by tangents to the posterior and inferior borders of the mandible. Planes—S–N, a transverse plane through the skull represented on a lateral skull radiograph tracing by a line joining sella and nasion; MxP, maxillary plane: a transverse plane through the skull represented on a lateral skull radiograph tracing by a line joining ANS and PNS which indicates the inclination of the maxilla; MnP, mandibular plane: a transverse plane through the skull represented on a lateral skull radiograph tracing by a line representing the lower border of the horizontal ramus of the mandible, constructed by joining Me and Go.

and the distance from the maxillary plane to Go. TAFH was measured by summing the perpendicular distance from the maxillary plane to Me and from nasion to the maxillary plane. These five measurements were selected as they were considered to exhibit the most consistent variation from normal when classifying vertical facial form (Schendel *et al.*, 1976; Opdebeeck *et al.*, 1978; Fields *et al.*, 1984).

The mean values for parameters SNMnP, MMPA, ArGoMe, and LAFH per cent were taken from Bhatia and Leighton (1993) and age matched to those of the experimental subjects. If the subject's values fell between the mean value ± 1 SD, then that parameter was categorized as normal. With the exception of PFH/TAFH per cent, if the values were above that range the subject was classified as long-face. The normal range for PFH/TAFH is 62–65 per cent (Jarabak and Fizzell, 1972), and if a subject had was value of 60 per cent or less, then this parameter was defined as long. A subject was classified overall as having a normal or long face if the balance of the parameters were of that type.

Preparation of biopsy specimens

All biopsy specimens were embedded in optimum cutting temperature embedding compound (OCT, H&E Ltd, Nottingham, UK) on a cork disc. The tissue was snap frozen in thawing isopentane (BDH, Poole, Dorset, UK) that had previously been frozen in liquid nitrogen at –196°C, and subsequently stored in an airtight polythene container at –70°C until required for sectioning. The masseter muscle is a highly pennate muscle and there is some evidence to suggest that biopsy samples can be quite variable in terms of connective tissue content (Thornell *et al*., 1984). In order to ensure that this was not a confounding factor in the experiments, a single section serial to those taken for MMP and TIMP analysis was stained using standard histological techniques (haematoxlylin and eosin; H&E) and the percentage of connective tissue determined by image analysis. Subsequent microscopic images were then analysed using the public-domain NIH Image J program (Abramoff *et al.*, 2004).

Detection and measurement of MMP-2 and MMP-9 by gelatinase zymography

Twenty 10 μm thick cryostat sections of frozen biopsies were cut and homogenized in Tris buffer (50 mM Tris–HCl, pH 7.5), containing 75 mM $CaCl_2$ (all reagents from BDH; Rao *et al.*, 1993). Samples were centrifuged at 5000 *g* for 20 minutes and the supernatant was used for MMP-2 and MMP-9 analysis. A protein assay (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) was used to measure the protein concentration for each sample, and the equivalent 40 μg protein was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), under non-denaturing conditions, through a 4 per cent polyacrylamide stacking gel and in a 10 per cent separating gel containing 0.1 per cent gelatin (Bloom 300, Sigma, Poole, Dorset, UK). On completion of electrophoresis, the gels were washed twice, for 15 minutes, in 2.5 per cent (v/v) Triton X-100 (Sigma) and incubated at 37°C for 24 hours in 100 ml of 50 mM Tris–HCl, pH 7.6, 5 mM $CaCl_2$, 200 mM NaCl, and 2.5 per cent (v/v) Triton X-100. Gels were stained with 0.5 per cent Coomassie brilliant blue G-250 (Sigma) and dissolved in 30 per cent methanol/10 per cent acetic acid for approximately 3 hours. The gels were then destained in 45 per cent methanol/10 per cent acetic acid and air-dried using the GelAir Drying System (Bio-Rad) for 40 minutes. Gelatinase enzymes were visualized as cleared areas against the blue background of the gelatin gel and characterized by comparison with a molecular weight marker (Kaleidoscope prestained standards, Bio-Rad) and with control standards of recombinant human MMP-2 and MMP-9 (Oncogene Research Products, Calbiochem, Nottingham, UK). The gels were scanned and visualized using Alpha Imager™ (Alpha Innotech Corporation, San Leandro, California, USA). Quantification of MMP activity was carried out using NIH imaging. The intensity of the band produced by the known amounts of MMP-2 or MMP-9 standard was compared with those generated from the patient samples to allow quantification.

Detection and measurement of TIMP-1 and TIMP-2 by reverse zymography

Preparation of the samples was as for the zymography. Supernatants containing equal amounts (20–30 μg) of protein were used for TIMP analysis. The samples were separated by SDS-PAGE using 12 per cent gels containing 1 mg/ml of gelatin (Bloom 300, Sigma) and 40 μl activated human recombinant MMP-2 purified from mammalian cells (Oncogene Research Products). Gels were washed twice, for 15 minutes, in 2.5 per cent (v/v) Triton X-100 (Sigma) and incubated at 37°C for 48 hours in 100 ml of 50 mM Tris–HCl, pH 7.6, 5 mM $CaCl_2$, 200 mM NaCl, and 2.5 per cent (v/v) Triton X-100. Gels were stained with 0.5 per cent Coomassie brilliant blue G-250 (Sigma) and dissolved in 30 per cent methanol/10 per cent acetic acid, for approximately 48 hours at 23°C. The gels were then destained and dried as for zymography. TIMPs were determined by their discrete inhibition of MMP activity and visualized as a dark band on a lighter background. TIMPs were identified and characterized by comparison with a molecular weight marker (as for zymography) and with control standards of recombinant bovine TIMP-1 and human recombinant TIMP-2 (Oncogene Research Products). The gels were scanned as for zymography and quantification of TIMP activity was assessed using Phoretix 1D gel analysis software (Phoretix International, Newcastle-upon-Tyne, UK). The intensity of the band produced by the known amounts of TIMP-1 or TIMP-2 standard was compared with those generated from the patient samples to allow quantification.

Statistical analyses

All statistical analyses were performed using the Statistical Package for Social Sciences (version 12.0; SPSS Inc., Chicago, Illinois, USA). Spearman's rho correlation coefficients were calculated between MMP-2, MMP-9, TIMP-1, TIMP-2, and the cephalometric variables. Mann–Whitney *U*-tests were performed to compare the mean values of MMP and TIMP expression in the normal- and long-face groups.

Measurement reliability

All cephalometric points and measurements were performed by one author (HLT) and repeated a minimum of 1 month following the original tracing. Single intraclass correlation coefficients were calculated for the following measurements: MMPA (0.93), SNMnP (0.92), ArGoMe (0.96), UAFH (0.92), LAFH (0.99), upper posterior face height (0.83), lower posterior face height (0.95) and showed a high level of agreement.

Table 1 Cephalometric measurements derived from 20 subjects whose samples were used for MMP analysis.

Biopsy number	Gender	MMPA (°)	SNMnP (°)	ArGoMe (°)	LAFH (%)	PFH/TAFH (%)	Category
139	M	32	39	130	58	60	L
140	F	38	46	139	59	58	L
153	F	35	45	144	59	60	L
171	F	39	38	133	59	61	L
172	M	25	38	127	57	66	N
175	M	25	31	131	56	66	N
176	F	27	31	114	57	65	N
177	F	32	35	132	58	84	N
178	M	30	35	129	58	63	N
179	F	29	31	130	55	69	N
180	F	41	49	127	57	58	L
181	M	29	33	134	62	62	N
183	F	36	48	125	55	56	L
185	F	39	46	135	54	59	L
186	F	28	39	139	57	62	N
187	F	25	30	133	60	66	N
188	F	28	42	133	59	68	L
193	F	38	45	130	58	59	L
194	F	41	48	142	58	55	L
217	M	21	31	128	58	69	N

Table 2 Cephalometric measurements derived from 20 subjects whose samples were used for TIMP analysis.

Biopsy number	Gender	MMPA (°)	SNMnP (°)	ArGoMe (°)	LAFH (%)	PFH/TAFH (%)	Category
80	F	30	40	120	55	64	N
86	F	29	40	139	54	60	L
105	M	23	35	125	55	62	N
139	M	32	39	130	58	60	L
142	F	34	42	140	59	60	L
143	F	41	50	133	59	53	L
171	F	39	38	133	59	61	L
172	M	25	38	127	57	66	N
178	F	30	35	129	58	63	N
180	F	41	49	127	57	58	L
185	F	39	46	135	54	59	L
188	F	28	40	133	59	68	L
193	F	38	45	130	58	59	L
207	M	28	32	127	59	72	N
209	F	28	41	133	54	59	L
214	M	23	32	136	57	66	N
215	F	25	39	135	54	59	N
219	F	30	40	134	57	61	N
224	F	21	28	118	54	69	N
226	F	26	33	131	56	67	N

F, female; M, male; MMPA, maxillary (M)–mandibular plane (MP) angle; SNMnP, sella–nasion (SN)–mandibular plane (MnP) angle; ArGoMe, gonial angle; LAFH, lower anterior face height; PFH/TAFH, the ratio of posterior face height (PFH) to total anterior face height (TAFH); N, normal facial dimensions; L, long facial dimensions.

Results

Cephalometric analysis

According to the cephalometric analysis already described, all subjects were allocated to a specific group, normal (N) or long (L), depending on their vertical facial form. Equal representation of facial type was present in both the MMP (Table 1) and the TIMP (Table 2) investigations.

Connective tissue content of biopsy samples

To ensure that the observations were not confounded by large variations in the amount of connective tissue sampled, sections parallel to those used for MMP and TIMP analysis were histologically stained and the percentage of connective tissue was determined by image analysis (Figure 2). In sections derived from the muscle of subjects with a normal facial form, the connective tissue occupied 15.51 ± 5.92 (mean ± SD) per cent of the area of the section (n = 11), while in sections derived from the muscles of subjects with long faces, the connective tissue occupied 15.94 ± 3.44 per cent (n = 15). There was no statistically significant difference between these values; hence, variations in connective tissue content of the biopsy can be excluded as a contributory factor to the results.

Expression of gelatinase (MMP-2 and MMP-9) activity

Twenty samples (four replicates) were subjected to MMP-2 and MMP-9 analysis and representative samples are shown in Figure 3. MMP activity is seen as cleared areas in the dark background of the gels. Both MMP standards and molecular weight markers were used to identify the enzymes. Fifteen samples expressed MMP-2, but 12 of these were at very low (virtually non-detectable) levels. MMP-9 activity was also evident in 12 cases, but again in eight of these, it was almost undetectable.

Expression of TIMP-1 and TIMP-2 activity

A further 20 samples (minimum two replicates) were subjected to TIMP analysis and representative samples are shown in Figure 4. TIMP activity is seen as darker areas in the pale background of the gels. Both TIMP standards and molecular weight markers were used to identify the enzymes. Figure 5 illustrates the TIMP activity of all the samples analysed. All samples expressed TIMP-1, but only 13 expressed TIMP-2, and of these seven were virtually non-detectable. Figure 5 also includes the MMP activity of all the samples analysed (see above).

Proteinase activity and vertical facial form

Statistical analyses of the relationship between the five cephalometric variables in terms of proteinase activity are summarized in Table 3. There was no evidence of a significant correlation between any of the cephalometric variables and proteinase activity. Mann–Whitney U-tests demonstrated no significant difference in proteinase activity when comparing individuals with normal and increased vertical facial form (Table 4).

Discussion

This study consistently demonstrated TIMP-1 activity in human masseter muscle from subjects with normal facial form confirming earlier work showing the presence of TIMP-1 protein in this tissue. TIMP-2 activity, where expressed, was at a lower level. In addition, MMP-2 and MMP-9 activity, where expressed, was virtually non-detectable. The observations relating to MMP-2 and MMP-9 activity are in agreement with previous findings relating to MMP-2 and MMP-9 protein expression (Singh *et al.*, 2000). Under normal circumstances, expression of MMP-2

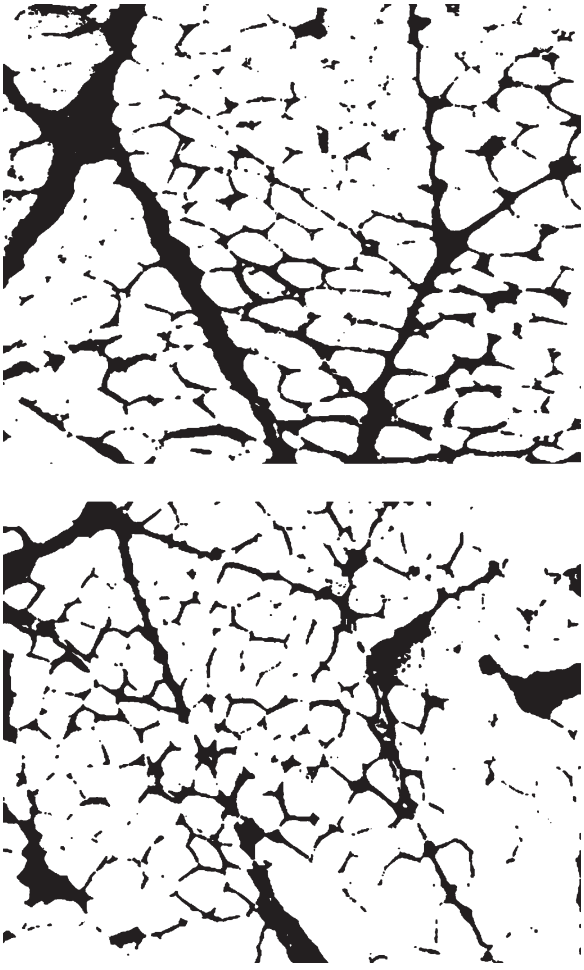

Figure 2 Histological sections of human masseter muscle derived from subjects with normal (upper panel) and long (lower panel) facial dimensions. These examples illustrate 'thresholding' the image with the connective tissue being black and the muscle fibres being white. The software then determines the number of back and white pixels in the field. The percentage of black is the amount of connective tissue. There are no obvious differences in the distribution of connective tissue between the two sections.

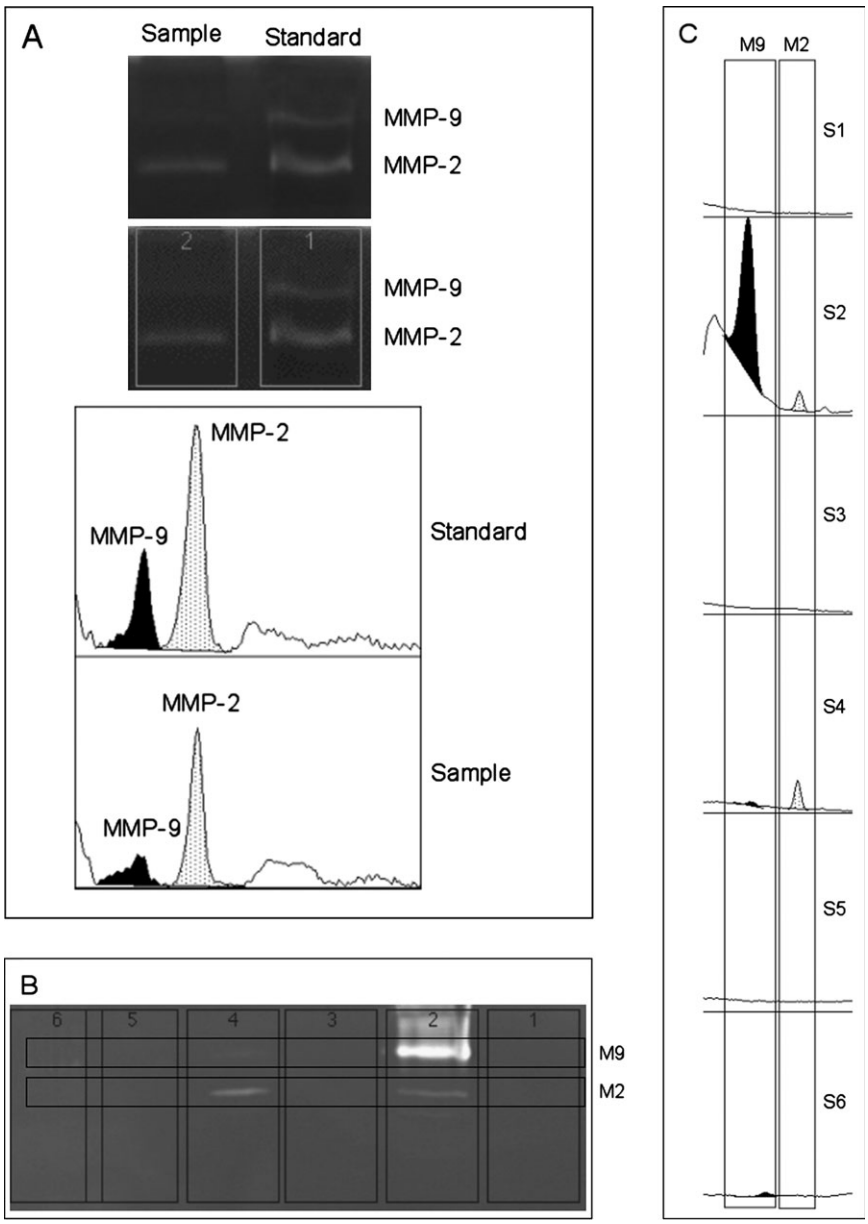

Figure 3 Gelatin gel zymogram for assessment of MMP-2 and MMP-9. (A) illustrates the method of quantification. Standard MMP was loaded onto each gel (Standard). Forty micrograms of protein from each sample was also loaded onto the gel. Following electrophoresis, images of gels were captured and using the software, lanes identified (1 and 2 on the upper panel). The software was then used to generate a density map (lower panel). The area under each peak was determined and the absolute amount of MMP determined by comparing the area under the peak for the sample with the area under the peak for the standard. This was expressed as the amount of gelatinase in nanograms. In (B), each lane (numbered) represents a single sample on a (representative) zymogram gel, while (C) represents the density traces for these bands. As can be seen on both the graphs and the gel, S1, S3, and S5 showed no detectable gelatinase activity; S2, S4, and S6 showed varying degrees of MMP-9 (M9) activity; and S2 and S4 showed varying degrees of MMP-2 (M2) activity.

and MMP-9 by skeletal muscle, including craniofacial muscle, is largely restricted to small amounts and confined to blood vessels, nerves, and neuromuscular junctions (Kherif *et al.*, 1999; Singh *et al.*, 2000). In contrast, during pathological and regenerative processes, or in response to experimentally injured muscle, MMP-2 and especially MMP-9 are upregulated (Kherif *et al.*, 1998, 1999; Schoser and Blottner, 1999).

ECM homeostasis, in the human masseter muscle, as in other systems, is a balance between secretion and synthesis of MMPs and their inhibition by TIMPs in a 1:1 manner. TIMP-1 and TIMP-2 activity has not been previously

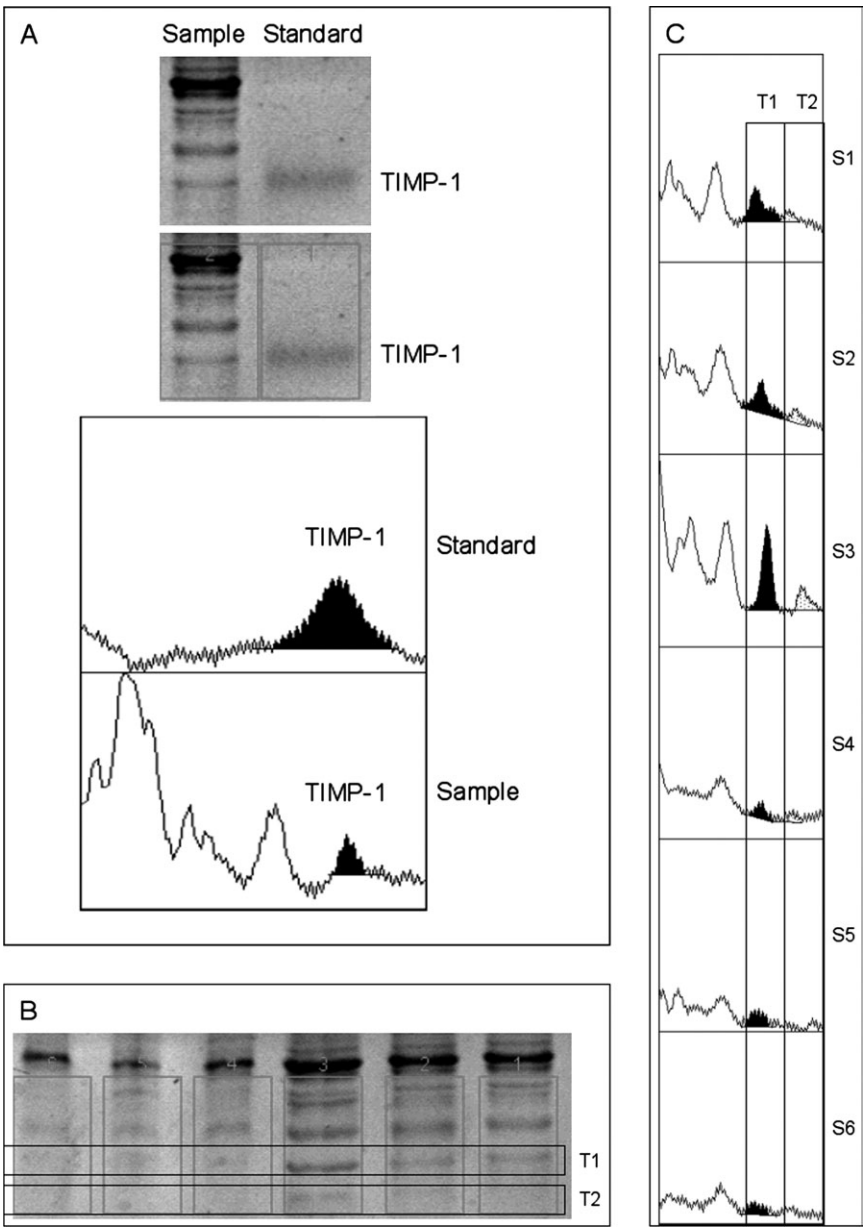

Figure 4 Gelatin gel reverse zymogram for assessment of TIMP-1 and TIMP-2. (A) illustrates the method of quantification. Standard TIMP-1 or TIMP-2 was loaded onto each gel (Standard). Forty micrograms of protein from each sample was also loaded onto the gel. Following electrophoresis, images of gels were captured and using the software, lanes identified (1 and 2 on the upper panel). The software was then used to generate a density map (lower panel). The area under each peak was determined and the absolute amount of TIMP determined by comparing the area under the peak for the sample with the area under the peak for the standard. This was expressed as the amount of TIMP in nanograms. In (B), each lane (numbered) represents a single sample on a (representative) reverse zymogram gel, while (C) represents the density traces for these bands. As can be seen on both the graphs and the gel, TIMP-1 bands are clearly seen for all the samples. S1, S2, S3, and S4 expressed TIMP-2 at low levels.

recorded in human craniofacial muscle but the consistent expression of TIMP-1 activity in all the samples investigated in this study (Figure 5) reflects the findings of an earlier study (Singh *et al.*, 2000), where TIMP-1 protein was clearly demonstrated in all samples of human masseter muscle. These data are confirmation of the previous suggestion (Singh *et al.*, 2000) that normal craniofacial jaw muscle has a relatively low turnover of connective tissue, part of which can be attributed to overwhelming levels of TIMP proteins (particularly TIMP-1) causing low activity of MMPs, particularly gelatinases. This is borne out by the low level of gelatinase activity observed in these samples.

The second aim of this study was to examine mediators of connective tissue remodelling in abnormal facial form. It is well documented that changes in muscle structure are observed in the jaw muscles of such subjects (Hunt *et al.*, 2006), probably a reflection of adaptive change. Equally, it is well established that dynamically changing muscle in other, non-craniofacial muscle, systems is associated with elevation of gelatinase expression, particularly MMP-9 (Kherif *et al*., 1998, 1999; Choi and Dalakas, 2000; Frisdal *et al.*, 2000). It might, therefore, be reasonable to expect that individuals with long-face syndrome (LFS), whose muscles show features of incomplete remodelling, may demonstrate a different profile of MMP and TIMP expression when compared with those individuals with normal facial form. These data clearly show a demonstrable variation in MMP and TIMP expression in different individuals (Figure 5). However, when the individuals were segregated in to two groups according to facial type, no discernible differences were found (Table 4). Furthermore, proteinase activity appeared to be unrelated to the five cephalometric measurements (Table 3). If LFS is considered as an extreme of normal variation, it may also be expected that changes in expression of ECM molecules would show more subtle variation when comparing LFS and normal vertical facial form. The findings are consistent with this theory.

Conclusion

This preliminary study, with its relatively small sample size, demonstrated individual variation in expression of ECM molecules but was unable to confirm that indices of ECM turnover are a reflection of an individual's vertical facial form although there was some suggestion that TIMP-1 might be related to facial form. Clearly, further research is required to establish the pathogenesis of LFS.

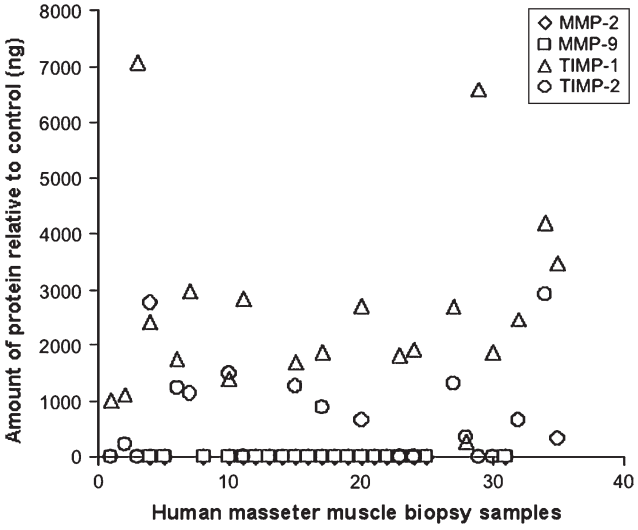

Figure 5 MMP and TIMP protein produced by human masseter muscle. MMP-2 was expressed in 15 samples, but of these, 12 were at a very low level. MMP-9 was also evident in 12 samples but in eight, as with MMP-2, was virtually undetectable. TIMP-1 was demonstrated in all samples investigated. TIMP-2 was expressed in 13 samples, and of these, seven were virtually undetectable.

Table 3 Correlation coefficients between chosen cephalometric measurements and proteinase activity in the human masseter muscle.

	MMP-2	MMP-9	TIMP-1	TIMP-2
SNMnP				
Spearman's rho correlation	−0.093	−0.262	−0.385	−0.100
Significance (two-tailed)	0.697	0.265	0.094	0.676
n	20	20	20	20
MMPA				
Spearman's rho correlation	−0.236	−0.252	−0.380	0.357
Significance (two-tailed)	0.317	0.284	0.098	0.122
n	20	20	20	20
ArGoMe				
Spearman's rho correlation	0.354	0.108	−0.216	−0.107
Significance (two-tailed)	0.126	0.650	0.361	0.652
n	20	20	20	20
LAFH (%)				
Spearman's rho correlation	0.416	0.149	−0.038	0.293
Significance (two-tailed)	0.068	0.53	0.875	0.209
n	20	20	20	20
PFH/TAFH (%)				
Spearman's rho correlation	0.017	0.019	0.276	−0.013
Significance (two-tailed)	0.944	0.938	0.239	0.956
n	20	20	20	20

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; MMPA, maxillary–mandibular plane angle; SNMnP, sella–nasion–mandibular plane angle; ArGoMe, gonial angle; LAFH, lower anterior face height; PFH/TAFH, the ratio of posterior face height to total anterior face height.

Table 4 Statistical analysis of proteinase activity in the human masseter muscle of normal and long-face individuals.

	Mann–Whitney
MMP-2 activity (normal versus long)	1.00
MMP-9 activity (normal versus long)	0.988
TIMP-1 activity (normal versus long)	0.075
TIMP-2 activity (normal versus long)	0.393

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase.

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