Coagulase-Negative Staphylococcal Membrane-Damaging Toxins, Pain Intensity, and Metabolic Changes in Temporomandibular Disorder Patients with Chronic Muscle Pain

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Aims: *To investigate the association between toxin-producing staphylococci, symptom expression, and changes in urinary excretion of metabolites in temporomandibular disorder (TMD) patients and age- and sex-matched control subjects.* ***Methods:*** *Twenty-nine patients defined by the research diagnostic criteria/TMD as having Type 1a muscle pain (TMD1A), and 34 age- and sex-matched control subjects were assessed for the carriage of staphylococcal species, staphylococcal toxin production, expression of symptoms, and changes in urinary excretion of amino and organic acids.* ***Results:*** *TMD1A patients had an increased incidence of carriage of toxin-producing coagulase-negative staphylococcus (MDT-CoNS, $P < .004$), which produced increased levels of δ-like membrane-damaging toxins. The TMD1A patients also had a reduction in the incidence of carriage of* Staphylococcus aureus *($P < .02$). Increased incidence of MDT-CoNS was positively associated with increased pain intensity as assessed by a visual analog scale ($P < .001$). Odds ratio analysis revealed a 9.2-fold increase in MDT-CoNS recovery from the nose of TMD1A patients compared with the control subjects (odds ratio = 9.2, > 95% confidence limits: 2.3 to 37.5, $P < .001$). Increases in the carriage incidence of MDT-CoNS were also associated with increases in the urinary tyrosine:leucine ratio ($P < .004$), which represents a change in the balance of proteolysis and protein synthesis. The toxin production by these CoNS species was also associated with an increased urinary excretion of glutamic acid ($P < .03$).* ***Conclusion:*** *These data suggest that an increased colonization of MDT-CoNS on skin and mucosal membranes was associated with changed proteolysis, increased pain intensity, and an increase in excitatory amino acids consistent with events associated with the development of chronic orofacial muscle pain in TMD patients.*

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Key words: facial pain, metabolism, microbiology, temporomandibular disorders, staphylococcus pathogenicity, staphylococcal haemolysins, staphylococcal toxicity

The most common pain disorder as defined by the research diagnostic criteria for temporomandibular disorders (RDC/TMD) is Type 1a muscle pain (TMD1A).[1] A report from our group confirmed that these patients are predominantly female, with most reporting events (infections, trauma, increased stress) at or around onset that were associated with alterations in host energy demand.[2] Chronic TMD1A pain patients have frequently reported general body symptoms of fatigue, muscle weakness, muscle fatigue, and symptoms involving musculoskeletal,

gastrointestinal, and genitourinary systems.[2] These patients also reported increased incidence of upper respiratory, gastrointestinal, and genitourinary infections at onset, and their sexual partners described an increased prevalence of chronic pain.[2] These data suggest that a systemic change occurs in TMD1A patients that may involve a pathogen-associated change.

An initial pilot study of such pain patients revealed an increased carriage of midstream urinary *Staphylococcus* spp without haematuria or pyuria.[3] A subsequent study[4] showed that these pain patients had an increased prevalence of multiple carriage of coagulase-negative staphylococci (CoNS), which produced membrane-damaging toxins (MDT-CoNS), compared with a pain- and symptom-free control group. Eighty-nine percent of the TMD1A pain patients were colonized by at least 1 CoNS producing either δ- or "horse"-haemolysin, and 75% had CoNS producing both types of haemolysin. In contrast, none of the symptom-free control subjects was colonized with CoNS that produced these membrane-damaging toxins. These data suggest that lipid-soluble staphylococcal membrane-damaging toxins could be an intrinsic component of chronic RDC/TMD Type 1a pain.[4] The TMD1A pain did not appear to be associated with the production of staphylococcal enterotoxins or toxic shock syndrome toxin-1, suggesting that pyrogenic toxins do not contribute to the etiology of chronic RDC/TMD Type 1a pain.[4]

Staphylococcal δ-toxins, which haemolyse human red blood cells, represent a group of highly lipid-soluble small polypeptides of low immunogenicity.[5] These proteins have a range of cytotoxic effects, including: *(1)* forming cation selective voltage-dependent and -independent ion channels,[6] *(2)* causing cell membrane lipid disturbances,[7–9] *(3)* causing cell permeability,[7] *(4)* reducing epidermal growth factor receptor function,[9] *(5)* activating phospholipase A2,[10] *(6)* inducing the production of prostaglandins and platelet-activating factor,[10] *(7)* acting as an enterotoxin,[11] and *(8)* inducing the production of cytokines.[12,13] In vitro experiments have demonstrated many of the attributes of δ-toxin, but very few studies have implicated these toxins as having clinical effects in vivo.[4,11,13] The "horse"-toxin has been reported but was unstable and could not be characterized.[14] However, analysis of the mechanisms of the action of δ-toxins associates them with potential dysregulation of proteolytic events within the host, including cytokine, cell volume, and growth hormone deregulation.

In their companion paper,[15] the authors have found that in the TMD1A patients: *(1)* there was an increase in the tyrosine:leucine ratio, which suggests elevated muscle catabolism in this group; and *(2)* leucine, which is a down-regulator of muscle catabolism, was substantially reduced in concentration. Increasing pain intensity was strongly associated with: *(1)* changes in the multivariate urinary excretion patterns, *(2)* diminished levels of leucine, *(3)* an increase in total urinary metabolites, and *(4)* increases in 2 unidentified urinary molecules coded UM28 and CFSUM1. TMD1A patients with the longest illness duration had: *(1)* the lowest urinary concentrations of metabolites, suggesting a progressive depletion of metabolite reserves throughout the illness, and *(2)* a progressive loss of succinic acid and combined glutamine + glutamic acid. These data partly address the hypothesis that: *(a)* chronic muscle pain conditions are characterized by repetitive acute exacerbations manifested as increases of pain intensity that are associated with the release of amino acids and other metabolites, and *(b)* increasing illness duration leads to an increase in pain distribution, symptom severity, and the development of symptoms within other tissues or organ systems. The authors further hypothesize that this appears to be a cytokine-mediate response to a number of challenges, particularly trauma and pathogen-induced changes. This study aims to address this hypothesis by investigating the association between toxin-producing staphylococci, symptom expression, and changes in urinary excretion of metabolites in RDC\TMD Type 1a patients and age- and sex-matched control subjects.

Methods

Pain Patient Selection

Forty-six patients sequentially presenting for orofacial pain management, with defined RDC/TMD1A muscle pain,[1] not associated with TMJ arthritis, sinusitis, dental, nerve, or vessel pathology, or any disease associated with pain, were recruited as previously described.[15] These patients were designated the TMD1A group.

Forty-one age- and sex-matched control subjects were identified (including relatives of the pain patients and unrelated subjects). Control subjects were eligible for inclusion if they had no response to pain on a visual analog scale (VAS) in the 2 weeks prior to consultation, did not give a history of chronic pain, and had not required professional

advice or treatment for chronic muscle pain in the previous 12 months.[15] Acute pain associated with trauma during the preceding 12 months was not an exclusionary criterion. Each subject provided informed consent in accordance with ethics requirements (Universities of Sydney and Newcastle) and was assessed by 1 clinician (NMcG).

Any medication being taken by a patient was recorded, and all subjects were asked to cease all medications apart from antidepressant and analgesic medications, as previously described.[15] Four study subjects were taking analgesics (3 TMD1A, 1 control), and 1 TMD1A patient was taking antidepressant medication. No patients had antibiotics within the previous 4 weeks.

Patient-reported muscle pain was confirmed by: *(1)* palpation of superficial facial, neck, and shoulder muscles; *(2)* a VAS (assessing muscle pain intensity); and *(3)* the responses to questions 1 (headaches), 12 (chest pain), 27 (low back pain), and 42 (muscle soreness) on a Hopkins Symptom Check List-90-Revised (SCL-90-R).[15]

The use of questionnaires, the calculation of indices, and urine specimens collection and Gas Chromatography Mass Spectrometry (GC-MS) identification, are described in detail in the companion paper.[15]

Sample Collection for Microbiology

After detailed instruction, control subjects and patients collected nasal swabs (transported in Stuart medium).[4]

Staphylococcal Haemolysin Assay

Haemolysins were assessed by haemolysis assays with the use of rabbit, sheep, horse, and human type O erythrocytes.[4] Supernatants from overnight growth of staphylococcal isolates were added to triple-washed erythrocytes of the 4 different types and incubated at 35°C for 30 minutes. The sheep erythrocyte suspensions were further refrigerated at 4°C for 24 hours to allow for the hot/cold haemolysis effect.[7] Samples were centrifuged at 3,500 rpm for 5 minutes and the absorbency (541 nm) was measured. The results were expressed as the percentage of the absorbency of a control tube containing totally lysed erythrocytes. The different haemolysis assays allowed detection of staphylococcal α-like (rabbit), β-like (sheep), γ-like (rabbit, sheep, and human), or δ-like toxin (human), and horse haemolytic toxin.[7,14]

A significant toxin-producing staphylococcus was defined as an isolate whose culture supernatant produced haemolysis at a level greater than or equal to 2 standard deviations from the mean haemolysis value produced by similar preparations from staphylococcal isolates, obtained from the pain/symptom-free control subjects.[4]

Statistical Analysis

The percentage composition urine data and the haemolysis data were arcsine-transformed before analysis. The urine concentration data were log-transformed to obtain normal distributions for regression analysis. Subject characteristics and symptom incidence were assessed by chi-square probability and *t* tests. Symptom indices, metabolites, and haemolysin data were compared by *t* test and forward stepwise multiple regression analysis. These data were processed by the use of Access 2000, Excel 2000, and Statistica.

Results

Patient Details

Initially, 46 TMD1A patients and 41 control subjects were recruited; 43 TMD1A and 40 control subjects completed all questionnaires and examination requirements.[3] Fourteen TMD1A patients and 6 control subjects were excluded, as urine samples were either not obtained (n = 16) or laboratory processing problems occurred (n = 4). The remainder comprised the study (n = 29) and control (n = 34) groups: age 38.2 ± 13 and 35.0 ± 13; gender characteristics 83% and 68% female; marital status 48% and 47% married, respectively.

Urinary Metabolites Excretion Patterns

Thirty-six urinary metabolites were detected and measured in this study. These data have been described previously,[15] with the significant differences including a reduction in the urinary output of leucine and increases in the relative output of tyrosine, UM28, and CFSUM1. UM28 and CFSUM1 were unidentified metabolites described in a previous study of chronic fatigue syndrome.[16] CFSUM1 was strongly associated with the severity of reported musculoskeletal symptoms, and positively correlated with increasing pain intensity in the whole group analysis but not within the TMD1A cohort.

Table 1 The Nasal Colonization Incidence of *Staphylococcal* spp and the Pain Intensity for TMD1A Patients (n = 29) and Control Subjects (n = 34)*

	TMD1A	Control	Pain Intensity		
	X(SE)	S (SE)	*P*	*r*	*P*
Nasal *Staphylococcus aureus*					
Total *S aureus*/subject	0.1 (0.4)†	0.4 (0.5)†	< .02	–0.28	< .03
Nasal coagulase negative staphylococci (CoNS)					
Total CoNS/subject	2.3 (1.0)†	1.8 (0.8)†	NS		NS
Total MDT-CoNS/subject	1.3 (0.7)†	0.7 (0.9)†	< .004	0.51	< .001
Discriminant function analysis					
Forward stepwise: TMD1A vs controls					
Wilks λ = .630, F = 7.820, *P* < .001					
Discriminant values					
1) MDT-CoNS	+				
2) CoNS species	+				
3) *S aureus*	–				
Multiple regression analysis					
Forward stepwise: pain intensity/bacterial parameters					
R^2 = 0.238, F = 9.042, *P* < .001					
Variables					
1) MDT-CoNS	+				
2) CoNS species	+				
3) *S aureus*	–				

*Statistical methods: Spearman rank-order correlation, Discriminate function and multiple regression analyses. α = *P* < .05. NS = not significant.
r = Spearman rank-order correlation; X = mean value; SE = standard error; R^2 = total variance of the dependent variable as explained by the independent variables (maximum 1.0). + = positive association; – = negative association; Wilks λ = ratio of the determinant of the within-groups variance/covariance matrix over the determinant of the total variance covariance matrix; MDT-CoNS = mambrane damaging toxin producing coagulase negative staphylococcus.
†The staphylococcus counts are expressed as the number of species detected per swab.

Staphylococcal Colonization in TMD1A Patients

TMD1A patients and control subjects were screened for the carriage of staphylococcal species, and each isolate was assessed for the ability to produce membrane-damaging toxins (Table 1). Control subjects had a higher incidence of carriage of *Staphylococcus aureus* compared with the TMD1A patients. In contrast, TMD1A patients had a higher carriage of CoNS that produced significant levels of membrane-damaging toxins (MDT-CoNS), compared with the control subjects. Since there were no differences in the total number of staphylococcal isolates detected in both clinical groups, it was concluded that there was a significant alteration in the distribution of the staphylococcal population in the TMD1A patients. These changes in bacterial flora reflect a shift toward an increased colonization of MDT-CoNS in the TMD1A group.

Multivariate analyses were used to assess whether the "commensal" distribution parameters were significantly different between TMD1A patients and control subjects. Discriminant function analysis revealed that the TMD1A patients and control subjects had different staphylococcal profile characteristics ($P < .001$, Table 1) and that the primary discriminant variable was the number of MDT-CoNS species per subject ($P < .001$). The odds of MDT-CoNS being recovered from the noses of TMD1A patients was 9.2-fold higher than in the control subjects (odds ratio: 9.2, 95% confidence limits: 2.3 to 37.5, $P < .001$).

Toxin-Producing Staphylococcus and Pain Intensity

The TMD1A patients had a mean pain intensity (VAS) score of 5.4 (95% confidence limits: 4.5 to 6.3); control subjects had a score of zero. Multiple regression analysis of the total study population (n = 63) revealed that increasing pain intensity scores were associated with alterations in the incidence of "commensal" staphylococcal isolates ($P < .001$), and that the primary correlate for pain intensity was the incidence of MDT-CoNS per subject

(Table 1). Univariate (Spearman rank) correlation analysis showed that increasing pain intensity was positively associated with the incidence of MDT-CoNS ($P < .001$). Conversely, increasing pain intensity was negatively associated with the number of *S aureus* isolates per subject. Figure 1 shows the association between the number of MDT-CoNS and pain intensity. A positive correlation was identified between pain intensity and "horse"-toxin production by CoNS ($r = 0.35$, $P < .006$), whereas α-toxin ($r = –0.26$, $P < .04$) and β-toxin ($r = –0.39$, $P < .002$) production by CoNS were negatively correlated with pain intensity.

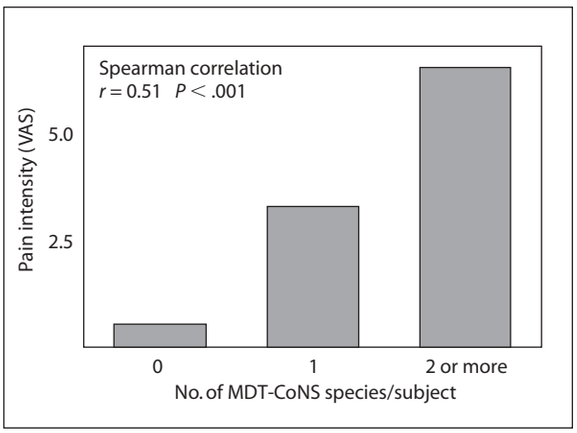

Fig 1 Association between the number of toxin-producing coagulase-negative staphylococcus species detected in the swab for each subject and pain intensity (VAS = 0 to 10 cm).

Toxin-Producing Staphylococcus and Urinary Metabolite Changes

Forward stepwise multiple regression analyses of the whole study population (n = 63; Table 2) revealed that the incidence of MDT-CoNS per subject was associated with alterations in the concentration ($P < .003$) and relative abundance ($P < .002$) of urinary metabolite excretion profiles. UM28 was the primary correlate in both regression models. Univariate correlation showed strong positive correlations between increases in incidence of MDT-CoNS per subject and increased UM28 and tyrosine output. A negative association was observed between increased incidence of MDT-CoNS and leucine output. Consequently, carriage of MDT-CoNS was positively associated with an increase in the tyrosine:leucine ratio (Table 2, Fig 2). Thus, CoNS α- and "horse"-membrane damaging toxicity is associated with an increased excretion of tyrosine (a marker of proteolysis) and a reduction in leucine (a marker of protein synthesis).

Forward stepwise multiple regression analyses of the TMD1A patient group (n = 29; Table 2) indicated a higher degree of association of MDT-CoNS with specific alterations in the concentration ($P < .003$) and relative abundance ($P < .008$) of the urinary metabolites. An unknown urinary metabolite, UM13a, together with combined glutamic acid + glutamine and aspartic acid were included amongst the 3 primary factors associated with the carriage of MDT-CoNS. Univariate correlation analyses revealed positive associations between the incidence of MDT-CoNS and glutamic acid/glutamine output in both concentration and relative abundance analyses. In the concentration analysis, positive correlations were also found for the carriage of MDT-CoNS with the following unknown urinary markers: UM15a, UM13a, UM27, and UM13. These data indicate a positive association between increases in the urinary excretion of an excitatory amino acid (combined glutamic acid + glutamine) and a series of unknown metabolites in the TMD1A patients, which may originate from the CoNS or reflect a biochemical host response.

Discussion

This study has identified an important association between carriage of MDT-CoNS and pain intensity and metabolic changes in TMD1A patients. While the carriage of different staphylococcal isolates for the TMD1A patients and the control subjects was similar, TMD1A patients had an increase in the carriage of MDT-CoNS that produce predominantly "horse"-14 and α-membrane-damaging haemolysins.[7] Multiple regression and correlation analyses associated the carriage of toxin-producing CoNS species with increased pain intensity. The highly significant odds ratio (9.2) for the carriage incidence of membrane-damaging toxin-producing staphylococcus between the TMD1A and control subjects suggests that TMD1A patients are more prone to the colonization of these organisms. There was a shift in the staphylococcal flora away from the coagulase-positive *S aureus* toward coagulase-negative staphylococcal species. These included *S epidermidis*, *S haemolyticus*, *S xylosis*, *S warneri*, *S hominis*, and *S lugdunensis*[4] as described in the previous paper,[4] where no association was found with any particular CoNS species and led to the grouping of all species under the heading of CoNS in this study. Why this shift in the microbial population occurs in these pain patients is unknown; however,

Table 2 Summary of the Urinary Excretion of Metabolites Significantly Correlated* with the Number of MDT-CoNS within the (a) Whole Study Group (n = 63) and the (b) TMD1A Group (n = 29)

	Concentration (mmol)		Relative Abundance	
	r	*P*	*r*	*P*
(a) Whole study group				
Positive correlations				
UM28	0.34	< .007	0.30	< .02
Tyrosine:leucine ratio	0.36	< .004	—	—
Negative correlations				
Leucine	–0.31	< .02	–0.32	< .02
Multiple regression analysis				
	R^2 = 0.325		R^2 = 0.420	
	F = 3.72		F = 3.29	
	P < .003		*P* < .002	
First correlates	(1) UM28	+	(1) UM28	+
	(2) hippuric acid	+	(2) UM14	+
	(3) UM15a	+	(3) 3-Methylhistidine	+
(b) TMD1A Group				
Postitive correlations				
Glutamic acid/glutamine	0.45	< .02	0.43	< .03
UM15a	0.44	< .02	—	—
UM13a	0.42	< .03	—	—
UM27	0.38	< .04	—	—
UM13	0.37	< .05	—	—
Multiple regression analysis				
	R^2 = 0.693		R^2 = 0.672	
	F = 4.76		F = 3.69	
	P < .003		*P* < .008	
First correlates	(1) UM13a	+	(1) glutamic acid	+
	(2) glutamic acid	+	(2) aspartic acid	–
	(3) aspartic acid	–	(3) leucine	–

*Statistical methods: Spearman rank-order correlation and multiple regression analyses. α = *P* < .05. *r* = Spearman rank-order correlation; R^2 = total variance of the dependent variable as explained by the independent variables (maximum 1.0). + = positive association; – = negative association.

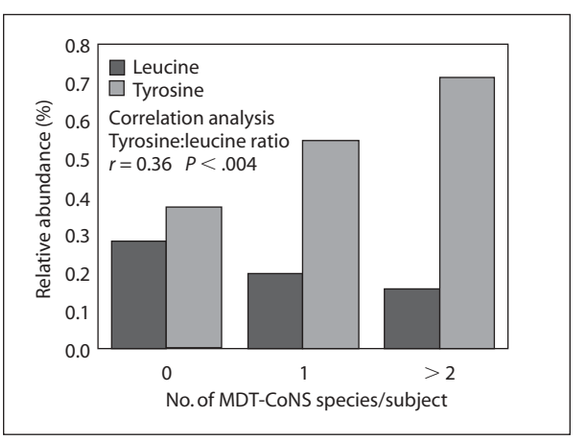

Fig 2 Association between the number of toxin-producing coagulase-negative staphylococcus species detected in the swab for each subject and the relative abundance of tyrosine and leucine in the whole study group (n = 63).

several possibilities exist. One possibility is that the toxin production by the organisms allows the CoNS species to predominate. Alternatively, prescription of antibiotics, which occurred in 13 of the TMD1A patients (data not shown), may facilitate an increase in either toxicity of these organisms or an exaggerated cytokine production to CoNS toxins[17] may also be significant in this selective process. Another intriguing possibility is that a change in host chemistry has facilitated the change in the microflora. In support of this possibility is the increase in catecholamines and their by-product metabolites that preferentially act as growth factors that select for CoNS species over *S aureus*.[18] Interestingly, an increase in urinary catecholamine excretion has been reported for TMD patients.[19] Stress has been implicated in the onset of TMD,[20] and in this study the urinary excretion of the catecholamine precursor, tyrosine, was positively correlated with increase

in carriage of MDT-CoNS. Further studies are required to assess these possibilities.

The TMD1A patients had reductions in the proteolysis-controlling amino acid, leucine, and alterations in individual amino acids associated with regulation of nonfibrillar proteolysis (serine, alanine, glycine, valine, threonine).[21–24] The urinary tyrosine:leucine ratio, an indicator of the ratio of proteolysis to protein synthesis, was elevated in the TMD1A patients. The increase in the tyrosine:leucine ratio and the reduction in leucine with increasing carriage of MDT-CoNS were consistent with an association between staphylococcal membrane-damaging toxin production and deregulated proteolysis. While past research indicated that reduced muscle total protein and RNA[25–27] was associated with either persistent enteroviruses within muscle[25] or reactivation of herpes simplex-627 in chronic fatigue syndrome patients, data from this study suggest that bacterial toxicity may also play a role in the chronic muscle pain in TMD patients. This association between bacterial toxicity and deregulated protein metabolism may also occur via an interferon-γ associated process,[28] which may result in a similar clinical outcome to that seen with viruses. Interestingly, *S epidermidis* isolates have been reported to produce symptoms in patients similar to toxic shock syndrome by inducing the production of cytokines (tumor necrosis factor, Interleukin-6, and Interleukin 1-beta).[13] This may be associated with an action by δ-toxin in conjunction with other staphylococcal exoproteins.[12] The unknown urinary marker, UM28, noted in the TMD1A patients, was strongly associated with the carriage incidence of MDT-CoNS and may prove to be a valuable diagnostic marker in the study of patients with chronic muscle pain. The fact that symptom-free control subjects[4] did not carry MDT-CoNS and had a tyrosine:leucine ratio of 1 further suggests that coagulase-negative staphylococcal membrane-damaging toxins were associated with deregulation of protein turnover and pain expression in these TMD patients.

In this study, MDT-CoNS were positively associated with pain intensity as well as increases in the urinary excretion of the excitatory amino acid, glutamic acid + glutamine in TMD1A patients. Coderre[29] proposed that increases in spinal fluid concentrations of the excitatory amino acids are required in the activation of the spinal cord N-methyl-D-aspartate (NMDA) receptor, which is associated with the induction of hyperalgesia. Increased sensitization of both peripheral nociceptors and nociceptive neurons in the central nervous system is also attributed to increases in the turnover of glutamic and aspartic acids.[29] The continual presence of a skin or mucosal membrane lipid-soluble, toxin-producing staphylococcus may provide a persistent stimulus leading to chronic pain. As a result, toxin-producing CoNS appear to comply with a number of the requirements for the development of chronic pain, such as a persistent stimulus, increased excitatory amino acid availability, and provision of a toxic stimulus that initiates an alteration in protein turnover (tyrosine:leucine ratio). This is also supported by the observation that these types of toxins are so small that they are unlikely to induce a protective antibody response.[5]

Conclusion

Although it is recognized that the present study's sample size is small, the data provide a basis for a new hypothesis on the etiology of chronic muscle pain disorders whereby lipid-soluble membrane-damaging toxins produced by variants of normal skin and mucosal surface coagulase-negative staphylococcal species are associated with pain intensity and deregulation of protein turnover. This study suggests that membrane-damaging toxin-producing microorganisms may be associated with the development of chronic RDC/TMD Type 1a pain, and that their removal may provide a specific treatment for chronic RDC/TMD Type 1a pain patients. Further studies are required to understand these proposed associations.

Acknowledgments

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