Université de Lille

École doctorale biologie santé

INSERM U1008: Advanced Drug Delivery Systems

# Prise en charge personnalisée et mini-invasive des dysfonctions temporomandibulaires

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Habilitation à diriger des recherches

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Devant un jury composé de :

Professeur Joël Ferri, président du jury

Professeure Isabelle Barthélémy, rapportrice

Professeure Muriel Brix, rapportrice

Professeur Pierre Corre, rapporteur

Professeur Gwénaël Raoul, examinateur

Rapporteur interne :

Professeur Patrick Vermersch

« Après un marathon, on sait davantage ce que l'on cherche dans l'existence. »

Paula Radcliffe, world marathon record holder

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Madame Marion Constant (Association des polymorphismes d'ENPP1 et du remodelage osseux de l'articulation temporomandibulaire dans une population de patients présentant une dysmorphose dento-maxillo-faciale ; thèse de médecine publiée en 2017 dans Journal of Craniomaxillofacial Surgery)

Madame Fanny Bruguière (Parafonctions, dysfonctions orofaciales et dysfonctions temporomandibulaire en chirurgie orthognathique. Une étude prospective de cohorte ; thèse de médecine publiée en 2019 dans Journal of Oral Rehabilitation)

Madame Sidonie Villa (Examen clinique et paraclinique de l'appareil manducateur normal et dysfonctionnel ; publication didactique publiée en 2020 dans EMC Chirurgie orale et maxillo-faciale)

Monsieur Thomas Roland-Billecart (Le type d'ostéosynthèse influence-t-il la santé articulaire en chirurgie orthognathique ? ; thèse de médecine publiée en 2021 dans Journal of Stomatology, Oral and Maxillofacial Surgery)

Monsieur Florent Barry (Induction d'un modèle chimique d'ostéoarthrite chez le rat – Un essai comparant l'injection de Collagenase de type 2 versus Monoacetate iodosodique ; Master 2 biologie - santé - parcours precision health soutenu en 2021 et publié en 2023 dans Plos One)

Madame Marie Béret (Évaluation d'un hydrogel injectable stérilisé dans la prise en charge de l'ostéoarthrite temporomandibulaire. Étude des propriété viscoélastiques et expérimentation animale chez le rat ; - Master 2 Biomatériaux soutenu en 2022 en cours de publication)

*A Cerise, Eliott, et Augustin.*

*Mes essentiels.*

**Je dédie ce travail.**

# Liste des tableaux et figures

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## Résumé

Les dysfonctions temporomandibulaires constituent un groupe de pathologies hétérogènes dont la nosologie est complexe et a beaucoup évolué au travers des années. Elles incluent un ensemble de présentations cliniques avec des formes articulaires et musculaires souvent combinées entre elles et dont l'étiopathogénie est multifactorielle. Leur prise en charge est un défi pour les professionnels de santé, principalement du fait de leur grande hétérogénéité et de l'évolution des concepts au travers du temps. Autrefois purement occlusale, leur prise en charge thérapeutique a progressivement migré vers une approche multidisciplinaire basée sur le modèle biopsychosocial de la douleur. Elle comprend des traitements conservateurs, des traitements mini-invasifs et des traitements chirurgicaux dont les modalités dépendent de la forme nosologique dysfonctionnelle et des facteurs étiopathogéniques identifiés.

La première partie de cette HDR rapporte la constitution d'une collection biomédicale réalisée sur une cohorte de patients devant bénéficier d'une prise en charge orthodontico-chirurgicale dans le cadre de la prise en charge d'une dysmorphose dentofaciale. L'objectif général de cette cohorte était d'étudier le génotype de gènes d'intérêt et le phénotype musculaire massétérin et d'étudier leur interrelation dans la genèse des dysmorphoses dentofaciales, des dysfonctions temporomandibulaires et du bruxisme. Nous nous sommes notamment intéressés à l'étude de polymorphismes nucléotidiques des gènes PITX1&2, ESR1, ENPP1 et ACTN3.

Dans la seconde partie de ce travail nous avons cherché à développer une technique mini-invasive dans la prise en charge de ces dysfonctions temporomandibulaires sous la forme d'un hydrogel injectable. Nous avons mis en place un modèle animal d'ostéoarthrite temporomandibulaire chez le rat, puis nous avons développé et testé un hydrogel injectable à base de Chitosan et polymères de Cyclodextrine avec libération prolongée de substance pharmacologique active. Nous présentons dans ce document les étapes de création du modèle animal et les analyses relatives aux principaux comparateurs.



**Mots-clés** : Dysfonction temporomandibulaire ; Dysmorphose dentofaciale ; Alfa-actinine 3 ; Muscle masséter ; Bruxisme ; Système de délivrance de médicament ; Modèle animal ; Ostéoarthrite temporomandibulaire ; Thérapies mini-invasives ; Toxine botulique A

## Abstract

Temporomandibular disorders constitute a group of heterogeneous pathologies whose nosology is complex and has evolved considerably over the years. They include a range of clinical presentations with articular and muscular forms that are often combined and whose etiopathogeny is multifactorial. Their management is a challenge for health professionals, mainly because of their great heterogeneity and the evolution of concepts over time. Formerly purely occlusal, their therapeutic management has progressively migrated towards a multidisciplinary approach based on the biopsychosocial model of pain. It includes conservative, minimally invasive and surgical treatments, the modalities of which depend on the dysfunctional nosological form and the etiopathogenic factors identified.

The first part of this work aimed to establish a biomedical collection on a cohort of patients benefiting from orthodontic-surgical management in the context of dentofacial deformities. The general objective of this cohort was to study the genotype of genes of interest, the masseteric muscle phenotype and their interrelationship in the genesis of dentofacial deformities, temporomandibular disorders and bruxism. We were particularly interested in the study of nucleotide polymorphisms of the PITX1&2, ESR1, ENPP1 and ACTN3 genes.

In the second part of this work, we aimed to develop a minimally invasive technique for the treatment of temporomandibular disorders in the form of an injectable hydrogel. We set up an animal model of temporomandibular osteoarthritis in rats, then we developed and tested an injectable hydrogel based on Chitosan and Cyclodextrin polymers with sustained drug delivery system. We present the steps for creating the animal model and the analyses for the main comparators.



**Keywords:** Temporomandibular disorders; Dentofacial deformity; Alfa-actinin 3; Masseter muscle; Bruxism; Drug-delivery system; Animal Model; Temporomandibular-joint osteoarthritis; Minimally invasive therapies; Botulinum Toxin A

# Curriculum Vitae

## I. Formation théorique

### 1. Cursus médical

- Qualification ordinale en Chirurgie orale 2019
- DESCQ de stomatologie et chirurgie maxillo-faciale (Lille) 2017
- DES de chirurgie générale (Lille) 2015
- Diplôme d'État de docteur en médecine (Lille) 2015

### 2. Cursus Scientifique

- Doctorat Biologie Santé : Biomolécules, Pharmacologie, Thérapeutique (Lille) / Rôle des Génotypes d'ACTN3 dans l'équilibre de l'appareil manducateur (Advanced Drug Delivery Systems - U1008) 2021
- Lauréat au prix « Prix de l'Innovation Pédagogique en Chirurgie » de l'Académie Nationale de Chirurgie 2020
- Prime d'encadrement doctoral et de recherche 2019/2023
- Diplôme d'État de docteur en médecine (Lille) / Thèse d'exercice médical : Symptomatologie dysfonctionnelle après chirurgie orthognathique : rôle du polymorphisme des gènes ACTN3, ENPP1, ESR1, PITX1 et PITX2 2015
- Master 2 Recherche Biologie Santé : Génomique, Génétique et Microbiologie (Lille) / Régulation des gènes de la voie Nodale dans l'asymétrie faciale (Advanced Drug Delivery Systems - U1008) 2014
- Master 1 Biologie Santé : Oncogenèse et différentiation / Génétique des populations (Paris 12) 2009

### 3. Formations complémentaires

- AUEC – Formation spécifique destinée aux personnes concevant et réalisant ou appliquant les procédures d'expérimentation animale (Lille) 2019
- Diplôme Inter-Universitaire « Pédagogie en sciences de la santé » (Rouen, Paris) 2018
- Certification Citi Program course: Human Subjects Research 2017

- Formation au Snap-Freezing et à l'histomorphométrie des muscles striés au Centre for Human & Applied Physiological Sciences, King's College London par le Dr A. Rowlerston 2012

## II. Formation pratique

- Référent médical de la plateforme d'Impression 3D enseignement/recherche de l'UFR3S dans le cadre du CPER TecSante depuis 2021

- Mobilité recherche - Laboratoire de Mécanique, Multiphysique, Multiéchelle (LaMcube), UMR 9013, Centrale Lille) / ReATM-Mini : Remplacement Articulaire TemporoMandibulaire Mini-invasif – Conception assistée par ordinateur, Simulation par méthode des éléments finis, Python, Réalité virtuelle augmentée, Impression 3D métal 2022

- Maître de Conférence Universitaire – Praticien Hospitalier - Service de chirurgie maxillo-faciale et stomatologie, CHU Lille (Pr Ferri) ; Unité Inserm U1008, Advanced Drug Delivery Systems (Pr Siepmann) depuis 2018

- Chef de Clinique Universitaire - Assistant des Hôpitaux - Service de chirurgie maxillo-faciale et stomatologie / Pr Ferri (CHU Lille) 2015/2018

# Valorisation scientifique en rapport avec l'HDR

## I. Encadrements de travaux de recherche

### 1. Encadrements de Thèses d'Université et Directions de Master 2

#### *A. Encadrements de Thèses d'Université*

- Modèles animaux des dysfonctions temporomandibulaires – Dr Florent Barry / EDBSL Lille (2022-2025)
- Appli'Dent – Conception et implémentation d'une application Smartphone dans le parcours de soins des Oligodonties – Ludovic Lauwers / EDBSL Lille (2020-2023)

#### *B. Directions de Master 2*

- Évaluation d'un hydrogel injectable stérilisé dans la prise en charge de l'ostéoarthrite temporomandibulaire. Étude des propriété viscoélastiques et expérimentation animale chez le rat. – Master 2 Sciences du médicament et des produits de santé (Lille) / Dr Marie Béret (2022)
- VolOrb3D : Volumétrie Orbitaire par impression 3D – Master 2 Sciences chirurgicales et nouvelles technologies interventionnelles (Université Paris-Saclay) – Mathilde de Massary (2022)
- Evaluation of a rat model with chemical induced temporomandibular osteoarthritis suitable for the study of a prolonged drug delivery system – Master 2 Biology and health science – Dr Florent Barry (2021)

### 2. Encadrements de Thèses e Médecine soutenues en rapport avec l'HDR

- Parafonctions, dysfonctions orofaciales et dysfonctions temporomandibulaire en chirurgie orthognathique. Une étude prospective de cohorte – Fanny Bruguière (2019)
- Amélioration de la qualité de vie après injections de toxine botulique A dans la prise en charge des dysfonctions temporomandibulaires – Sidonie Villa (2018)
- Le type d'ostéosynthèse influence-t-il la santé articulaire en chirurgie orthognathique ? – Thomas Roland-Billecart (2018)
- Association des polymorphismes d'ENPP1 et du remodelage osseux de l'articulation temporo-mandibulaire dans une population de patients présentant une dysmorphose dento-maxillo-faciale – Marion Constant (2016)

## II. Publications scientifiques en rapport direct avec l'HDR

- Beret M, Barry F, Chai F, Chijcheapaza-Flores H, Garcia-Fernandez MJ, Blanchemain N, **Nicot R**. Efficacy of intra-articular injection of botulinum toxin type A (incobotulinumtoxinA) in temporomandibular joint osteoarthritis: a three-arm controlled trial in rats. Article Soumis.

- Barry F, Chai F, Chijcheapaza-Flores H, Garcia-Fernandez MJ, Blanchemain N, **Nicot R**. Comparison of chemical-induced temporomandibular osteoarthritis rat models (monosodium iodoacetate versus collagenase type II) for the study of prolonged drug delivery systems. PLoS One. 2023.

- **Nicot R**, Raoul G, Vieira AR, Ferri J, Sciote JJ. ACTN3 genotype influences masseter muscle characteristics and self-reported bruxism. Oral Dis. 2023;29(1):232-244. doi: 10.1111/odi.14075.

- Barry F, Chai F, Chijcheapaza-Flores H, Garcia-Fernandez MJ, Blanchemain N, **Nicot R**. Systematic review of studies on drug-delivery systems for management of temporomandibular-joint osteoarthritis. J Stomatol Oral Maxillofac Surg. 2022;123(5):e336-e341. doi: 10.1016/j.jormas.2021.08.003.

- **Nicot R**, Raoul G, Sciote JJ. Response to "sleep bruxism, wake bruxism, or both? The importance of their full reporting and diagnosis". Oral Dis. 2022 doi: 10.1111/odi.14334.

- **Nicot R**. Traitements conservateurs, mini-invasifs et chirurgicaux des dysfonctions temporomandibulaires. EMC Chirurgie orale et maxillo-faciale. 2022; Doi : 10.1016/S2352-3999(21)41481-0.

- **Nicot R**, Mattei L, Raoul G, Tiffreau V, Ferri J, Schlund M. Limitation d'ouverture buccale. EMC Chirurgie orale et maxillo-faciale. 2022; Doi : 10.1016/S2352-3999(21)42356-3.

- **Nicot R**, Ferri, J. Ostéotomies maxillomandibulaires : techniques chirurgicales et principales indications. EMC Techniques chirurgicales - Chirurgie plastique reconstructrice et esthétique. 2021; Doi : 10.1016/S1286-9325(21)42488-X.

- **R Nicot**, F Barry, H Chijcheapaza-Flores, MJ Garcia-Fernandez, G Raoul, N Blanchemain, F Chai. A Systematic Review of Rat Models With Temporomandibular Osteoarthritis Suitable for the Study of Emerging Prolonged Intra-Articular Drug Delivery Systems. J Oral Maxillofac Surg. 2021 Aug;79(8):1650-1671. doi: 10.1016/j.joms.2021.02.034.

- **Nicot R**, Roland-Billecart T, Schlund M. Pathologies non fonctionnelles de l'articulation temporomandibulaire. EMC Chirurgie orale et maxillo-faciale. 2021; Doi : 10.1016/S2352-3999(20)41482-7.

- Roland-Billecart T, Raoul G, Kyheng M, Sciote JJ, Ferri J, **Nicot R**. TMJ related short-term outcomes comparing two different osteosynthesis techniques for bilateral sagittal

split osteotomy. J Stomatol Oral Maxillofac Surg. 2021 Feb;122(1):70-76. doi: 10.1016/j.jormas.2020.03.018.

- **Nicot R**, Chung K, Vieira AR, Raoul G, Ferri J, Sciote JJ. Condyle modeling stability, craniofacial asymmetry and ACTN3 genotypes: Contribution to TMD prevalence in a cohort of dentofacial deformities. PLoS One. 2020;15(7):e0236425. doi: 10.1371/journal.pone.0236425.

- **Nicot R**, Raoul G, Ferri, J. Étiopathogénie des dysfonctions temporomandibulaires. EMC Chirurgie orale et maxillo-faciale. 2020; Doi : 10.1016/S2352-3999(20)41480-3.

- Villa S, **Nicot R**, Raoul G, Ferri, J. Examen clinique et paraclinique de l'appareil manducateur normal et dysfonctionnel. EMC Chirurgie orale et maxillo-faciale. 2020; Doi : 10.1016/S2352-3999(20)41479-7.

- **Nicot R**, Raoul G. Histologie des muscles manducateurs. EMC Chirurgie orale et maxillo-faciale. 2019; Doi : 10.1016/S2352-3999(19)87382-X.

- Bruguiere F, Sciote JJ, Roland-Billecart T, Raoul G, Machuron F, Ferri J, **Nicot R**. Pre-operative parafunctional or dysfunctional oral habits are associated with the temporomandibular disorders after orthognathic surgery: An observational cohort study. J Oral Rehabil. 2019;46(4):321-329. doi: 10.1111/joor.12749.

- Chung K, Richards T, **Nicot R**, Vieira AR, Cruz CV, Raoul G, Ferri J, Sciote JJ. ENPP1 and ESR1 genotypes associated with subclassifications of craniofacial asymmetry and severity of temporomandibular disorders. Am J Orthod Dentofacial Orthop. 2017;152:631-645. doi: 10.1016/j.ajodo.2017.03.024.

- Constant M., **Nicot, R**., Vieira A.R., Raoul G., Sciote J.J., Ferri J. Condylar geometry variation is associated with ENPP1 variant in a population of patients with dento-facial deformities. J Craniomaxillofac Surg. 2017;5:826-830. doi: 10.1016/j.jcms.2017.02.020.

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- JH Godel, BF Foley, **R Nicot**, MJ Horton, ER Barton, J Ferri, G Raoul, AR Vieira, JJ Sciote. Associations between ACTN3 and OPPERA pain-related genes in malocclusion. Molecular Pain 2014 10 (Suppl 1):P4

- Zebrick B, Teeramongkolgul T, **Nicot R**, Horton MJ, Raoul G, Ferri J, Vieira AR, Sciote JJ. ACTN3 R577X genotypes associate with Class II and deepbite malocclusions. Am J Orthod Dentofacial Orthop. 2014;146:603-11. doi: 10.1016/j.ajodo.2014.07.021.

- **Nicot R**, Hottenstein M, Raoul G, Ferri J, Horton M, Tobias JW, Barton B, Gelé P, Sciote JJ. Nodal Pathway Genes Are Down-regulated in Facial Asymmetry. J Craniofac Surg. 2014;25 :e548-55. doi: 10.1097/SCS.0000000000001076.

## III. Communications scientifiques en rapport direct avec l'HDR

- Chirurgie orthognathique et dysfonction temporo-mandibulaire : que savoir en 2023 ? **R Nicot** – 23èmes Les Assises Face et Cou - Cannes 2023

- Chitosan-based hydrogel for drug delivery and viscosupplementation treatment of temporomandibular joint disorders. Chijcheapaza-Flores H, Garcia-Fernandez MJ, **Nicot R**, Tabary N, Cazaux F, Chai F, Martel B, Blanchemain N - International workshop on Biomaterial Innovations – Cergy-Pontoise 2022

- Chirurgie des désordres internes temporomandibulaires **R Nicot** - 57ème Congrès de la Société Française de Stomatologie Chirurgie Maxillo-Faciale et Chirurgie Orale – Reims 2022

- Quand et comment faire une arthrocentèse ? **R Nicot** - 57ème Congrès de la Société Française de Stomatologie Chirurgie Maxillo-Faciale et Chirurgie Orale – Reims 2022

- Arthroscopie de l'ATM. **R Nicot** - Session de l'AO CMF au 57ème Congrès de la Société Française de Stomatologie Chirurgie Maxillo-Faciale et Chirurgie Orale – Reims 2022

- Chitosan-based hydrogel for drug delivery and viscosupplementation treatment of temporomandibular joint disorders Henry Chijcheapaza-Flores, Maria José Garcia-Fernandez, **Romain Nicot**, Nicolas Tabary, Frédéric Cazaux, Feng Chai, Bernard Martel, Nicolas Blanchemain - 20e Journée Cyclodextrines – Lille 2022

- Utilisation de la toxine botulique A dans les dysfonctions temporomandibulaires. **R. Nicot** - Merz Expert Meeting – Loos 2021

- Utilisation de la toxine botulique A dans la PEC des douleurs orofaciales en contexte carcinologique des VADS. **R. Nicot** - Merz Expert Meeting – Webinar 2021

- Évaluation d'un modèle de rat présentant une ostéoarthrite temporomandibulaire chimiquement induite pour l'étude d'un système à libération prolongé. F. Barry, F. Chai, **R. Nicot** (PRIX DU MEILLEUR POSTER DU 55ème CONGRES DE LA SFSCMFCO) - 56ème Congrès de la Société Française de Stomatologie Chirurgie Maxillo-Faciale et Chirurgie Orale – Besançon 2021

- Injections intra-articulaires, arthrocentèse et arthroscopie temporomandibulaire dans la prise en charge des désordres internes. **R. Nicot** - 56ème Congrès de la Société Française de Stomatologie Chirurgie Maxillo-Faciale et Chirurgie Orale – Besançon 2021

- Techniques de prise en charge mini-invasive des dysfonctionnements temporomandibulaires. **R. Nicot** - 56ème Congrès de la Société Française de Stomatologie Chirurgie Maxillo-Faciale et Chirurgie Orale – Besançon 2021

- ACTN3 Associations with Adult Bruxism and Muscle Phenotype. J.J. Sciote, **R. Nicot**, J. Ferri, A.R. Vieira, G. Raoul - The 99th General Session of the IADR, the 50th Meeting of the AADR and the 45th Meeting of the CADR – Virtual Experience 2021

- Chirurgie Orthognathique et SADAM. Réalités en 2020. **R. Nicot** - 6ème journées de l'Association Internationale de Médecine Orale et Maxillo-faciale - Lille 2020

- ATM et chirurgie orthognathique. **R. Nicot -** Les confluences - Lille 2019

- Sex and TMD Dependent Expression of COMT in Human Masseter. M. Horton, A. Vieira, J. Ferri, **R. Nicot**, G. Raoul, J.J. Sciote - 97Th General Session & Exhibition of the International Association for Dental Research - Vancouver 2019

- Craniofacial Asymmetry and Variations In Masseter Muscle Fiber Types H. Gray, J. Moore, **R. Nicot**, M. Horton, G. Raoul, J. Ferri, J.J. Sciote - 97Th General Session & Exhibition of the International Association for Dental Research - Vancouver 2019

- Évaluation du niveau de risque articulaire postopératoire en chirurgie orthognathique : suivi prospectif de cohorte. **R. Nicot -** Journée de la recherche clinique chirurgicale - Nominé au « Grand prix de la recherche clinique 2018 » de l'Académie de Chirurgie - Paris 2018

- Identification de génotypes d'intérêts dans le développement du concept de médecine personnalisée en chirurgie orthognathique. **R. Nicot**, J.J. Sciote, A.R. Vieira, J. Ferri, G. Raoul - 54ème Congrès de la Société Française de Stomatologie Chirurgie Maxillo-Faciale et Chirurgie Orale – Marseille 2018

- Association entre présence préopératoire d'une parafonction ou dysfonction orofaciale et la survenue d'une dysfonction temporomandibulaire après chirurgie orthognathique. F. Bruguière, T. Roland-Billecart, G. Raoul, J.J. Sciote, J. Ferri, **R. Nicot -** 54ème Congrès de la Société Française de Stomatologie Chirurgie Maxillo-Faciale et Chirurgie Orale - Marseille 2018

- La technique d'ostéosynthèse n'influence pas la santé articulaire après chirurgie orthognathique. T. Roland-Billecart, G. Raoul, J.J. Sciote, J. Ferri, **R. Nicot -** 54ème Congrès de la Société Française de Stomatologie Chirurgie Maxillo-Faciale et Chirurgie Orale - Marseille 2018

- Introduction to personalized medicine in orthognathic surgery to improve joints results. **R. Nicot**, J.J. Sciote, A.R. Vieira, J. Ferri, G. Raoul - 24th Congress of the European Association for Cranio Maxillo Facial Surgery - Munich 2018

- Improvement of TMJ Pain-Relative Symptoms After Orthognathic Surgery. T. Roland-Billecart, G. Raoul, J.J. Sciote, J. Ferri, **R. Nicot** - 24th Congress of the European Association for Cranio Maxillo Facial Surgery - Munich 2018

- Facial Asymmetry, Condyle Geometry and ACTN3 Genotype Increase TMD Prevalence. J. Sciote, M. Constant, **R. Nicot**, K. Chung, Raoul, J. Ferri, A. Vieira, G. Raoul - 96Th General Session & Exhibition of the International Association for Dental Research – London 2018

- Simplified posteroanterior cephalometric analysis reveals four classifications of craniofacial asymmetry. K. Chung, J. Sciote, **R. Nicot**, Raoul, J. Ferri, J. Godel, E. Spannhake - 47rd Annual Meeting & Exhibition of the American Association for Dental Research - Fort Lauderdale 2018

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- Prix StartAIR Hauts-de-France 2019: MaxilloGel = Use of a Chitosan hydrogel with a sustained release system for analgesic pharmacological substances in the management of temporomandibular disorders.

- ANR JCJC 2021: Intra-articular analgesic viscosupplementation based on CHITOsan and poly-cyclodextrin.

# Introduction

*Musculoskeletal Heritable Influences on Malocclusion*

Les dysmorphoses dentofaciales sont des anomalies de position des dents et des mâchoires, entre elles et par rapport à la base du crâne, qui affectent plus de 20% de la population générale. Leur prise en charge, orthodontique ou orthodontico-chirurgicale, est justifiée par des objectifs morpho-fonctionnels. La genèse de ces dysmorphoses dentofaciales est sous la dépendance de 3 principaux facteurs (Humphrey 1998; Mao et Nah 2004) :

- La croissance du couple musculosquelettique liée à la fonction manducatrice, et en particulier à la force musculaire. Cette dernière est intimement liée au phénotype musculaire (type de fibres musculaires, nombre et diamètre de ces fibres).
- Les facteurs génétiques, qui interviennent dans la détermination du phénotype musculaire et dans la croissance des pièces osseuses.
- Les dysfonctions et les parafonctions orales.

Les travaux précédemment menés par notre équipe de recherche sur la relation entre dysmorphoses dentofaciales et phénotype musculaire du muscle masséter ont mis en évidence une relation étroite entre la hauteur faciale et les fibres de type rapide, ainsi qu'une relation significative entre l'asymétrie mandibulaire et les fibres de type rapide (Rowlerson et al. 2005; Raoul et al. 2011; Sciote et al. 2012, 2013). Les dysfonctions temporomandibulaires sont des comorbidités fréquentes des asymétries mandibulaires et pourraient également répondre à un profil histomorphométrique spécifique. (Raoul et al. 2011; Sciote et al. 2013; Al-Moraissi et al. 2017a; Toh et al. 2021).

Il a été montré que *PITX2,* un gène effecteur de la voie Nodale qui joue un rôle déterminant dans le développement de l'asymétrie gauche-droite pendant l'embryogenèse (Yoshioka et al. 1998; Shen 2007), est exprimé dans les cellules satellites du muscle squelettique humain adulte (Knopp et al. 2013). *PITX1*, un autre membre de la famille de gènes *PITX*, produit une atrophie des fibres musculaires lorsqu'il est surexprimé dans le muscle masséter (Pandey et al. 2012). Ces résultats suggèrent que les gènes de la famille *PITX* et les gènes de la voie Nodale sont actifs dans le muscle squelettique humain adulte et peuvent être des facteurs clés dans le développement, la croissance et le maintien de l'asymétrie faciale.

Les avancées en matière de cartographie du génome humain ont permis d'identifier des gènes influençant la croissance sagittale des mâchoires (Xue et al. 2010; Doraczynska-Kowalik et al. 2017). *MYO1H*, un gène codant pour une protéine du sarcomère, unité fonctionnelle du muscle strié squelettique, est l'un de ces gènes d'intérêt (Tassopoulou-Fishell et al. 2012; da Fontoura et al. 2015; Sun et al. 2018; Cunha et al. 2019; Dehesa-Santos et al. 2021). Cependant, d'autres gènes d'intérêt codant pour des protéines sarcomériques pourraient également être impliqués dans la croissance des mâchoires, la régulation du phénotype massétérin, pouvant affecter à fortiori la santé articulaire temporomandibulaire. L'un d'entre eux, *ACTN3*, code pour l'alpha actinine 3, une protéine d'ancrage des myofibrilles du muscle squelettique qui influencent les propriétés contractiles et qui est exprimée uniquement dans les fibres rapides (North et al. 1999). Une mutation ponctuelle, résultant en l'absence de la protéine alpha actinine 3, conduit à une diminution de l'activité contractile rapide, une amélioration des performances d'endurance et une réduction de la masse osseuse et la densité minérale osseuse (Houweling et al. 2018).

D'autres gènes ont par ailleurs été identifiés comme gènes d'intérêt dans l'influence de la croissance des mâchoires et la santé articulaire temporomandibulaire. Plusieurs études ont par exemple mis en évidence une association significative entre des polymorphismes du gène *ESR1*, codant pour le récepteur α à l'œstrogène, et des symptômes de dysfonction temporomandibulaire (Kim et al. 2010; Quinelato et al. 2018), avec une dysfonction à type d'ostéoarthrite chez des femmes (Kang et al. 2007; Ribeiro-Dasilva et al. 2009; Liu et al. 2014) ou encore avec un désordre interne temporomandibulaire (Dalewski et al. 2020). *ENPP1*, un gène codant pour l'ectonucléotide pyrophosphatase / phosphodiestérase 1, est un l'un des régulateurs principaux du processus de minéralisation osseuse. Il code pour une ecto-enzyme transmembranaire qui hydrolyse le pyrophosphate inorganique et par conséquent inhibe la formation de l'hydroxyapatite. Cheung CL et al. (Cheung et al. 2009) ont mis en évidence par la cohorte *Framigham*, l'implication de certains polymorphismes nucléotidiques du gène *ENPP1* dans la densité minérale osseuse et la morpho-biométrie osseuse en faisant un autre gène d'intérêt dans l'influence de la croissance des mâchoires et la santé articulaire temporomandibulaire.

Notre travail a d'abord visé à constituer une collection biomédicale sur une cohorte de patients devant bénéficier d'une prise en charge orthodontico-chirurgicale dans le cadre de la prise en charge d'une dysmorphose dentofaciale. L'objectif général de cette cohorte était d'étudier la relation entre le génotype de gènes d'intérêt et le phénotype musculaire massétérin et d'étudier

leur interrelation dans la genèse des dysmorphoses dentofaciales, des dysfonctions temporomandibulaires et du bruxisme. Au niveau génotypique, nous nous sommes intéressés à l'étude de polymorphismes nucléotidiques des gènes *PITX1&2, ESR1*, *ENPP1* et *ACTN3*.

*Hydros Medical / MaxilloGel*

Les dysfonctions temporomandibulaires sont aujourd'hui organisées au travers d'un système de classification basé sur ce modèle biopsychosocial de la douleur, appelé Diagnostic Criteria for Temporomandibular Disorders (DC/TMD) (Schiffman et al. 2014), qui inclut notamment une évaluation physique correspondant à l'axe I et une évaluation du statut psychosocial et de l'incapacité liée à la douleur au sein de l'axe II. D'un point de vue taxonomique, cette classification différencie plusieurs sous-catégories avec en chefs de file des pathologies de l'articulation temporomandibulaire et des pathologies musculaires masticatoires. La prise en charge des affections articulaires temporomandibulaires inclut des traitements conservateurs, mini-invasifs, et chirurgicaux, dans une approche multimodale. Les traitements conservateurs sont multiples avec une prise en charge médicamenteuse de la crise aigüe douloureuse (AINS, myorelaxants), de la kinésithérapie oro-maxillofaciale, des traitements occlusaux et une prise en charge psychologique et/ou de gestion du Stress. Alors que de nombreux auteurs se sont intéressés au lien existant entre chirurgie orthognathique et santé articulaire temporomandibulaire, les résultats restent ambivalents concernant la place de ce type de traitement dans la prise en charge des dysfonctions temporomandibulaires (Al-Riyami et al. 2009a, 2009b; Bermell-Baviera et al. 2016; Al-Moraissi et al. 2017b). En revanche, une méta-analyse des essais thérapeutiques randomisés contrôlés comparant les différents traitements mis en œuvre dans les formes articulaires a confirmé le rôle significativement plus efficace des procédures mini-invasives par rapport aux traitements conservateurs, tant pour la réduction de la douleur que pour l'amélioration de l'ouverture buccale, à court terme (jusqu'à 5 mois) et à plus long terme (6 mois à 4 ans) (Al-Moraissi et al. 2019).

Ces techniques mini-invasives incluent des injections intra-articulaires, l'arthrocentèse et l'arthroscopie. De nombreux agents pharmacologiques ont été utilisés sous la forme d'injections intra-articulaires, seuls ou en adjonction à l'arthrocentèse ou l'arthroscopie, avec des niveaux de preuve et des indications variables. Les principaux sont l'acide hyaluronique, le Plasma riche en plaquettes (PRP) et les corticoïdes. Plusieurs revues systématiques de la littérature et méta-analyses (Goiato et al., 2016 ; Iturriaga et al., 2017 ; Liu et al., 2018 ; Haigler

et al., 2018 ; Moldez et al., 2018 ; Liu et al., 2020) ont montré une efficacité des injections d'acide hyaluronique intra-articulaires dans le contrôle des douleurs et l'amélioration fonctionnelle dans le traitement des DTM (Goiato et al. 2016; Iturriaga et al. 2017; Haigler et al. 2018; Liu et al. 2018, 2019; Moldez et al. 2018). Plus particulièrement, chez les patients présentant une arthropathie dégénérative, l'acide hyaluronique permet une réduction des douleurs (Liu et al. 2018) et une amélioration de l'ouverture buccale sur le court terme (Liu et al. 2018, 2020). Plusieurs revues systématiques de la littérature et méta-analyses (Haigler et al., 2018 ; Bousnaki et al., 2018 ; Liu et al., 2020 ; Zotti et al., 2019 ; Sakalys et al., 2019 ; Chung et al., 2019) ont évalué l'efficacité des injections de plasma riche en plaquettes (PRP) comme thérapeutique injectable des désordres internes temporomandibulaires (Bousnaki et al. 2018; Haigler et al. 2018; Chung et al. 2019; Liu et al. 2020; Sakalys et al. 2019; Zotti et al. 2019). L'utilisation de PRP, par injection ou associée à une arthrocentèse était plus efficace que l'arthrocentèse seule ou associée à l'acide hyaluronique (Chung et al. 2019; Sakalys et al. 2019; Zotti et al. 2019), en particulier dans les cas d'arthropathies dégénératives temporomandibulaires avec d'excellents résultats sur la réduction des douleurs (Bousnaki et al. 2018; Chung et al. 2019; Liu et al. 2020). Concernant les résultats fonctionnels les résultats sont plus mitigés, certaines revues systématiques de la littérature et méta-analyses retrouvant une amélioration (Bousnaki et al. 2018; Haigler et al. 2018; Liu et al. 2020; Zotti et al. 2019), et d'autres non (Chung et al. 2019; Sakalys et al. 2019). Concernant l'utilisation de corticoïdes injectables, une méta-analyse réalisée par Moldez et al. en 2018 n'a pas retrouvé de différence entre l'injection de corticoïdes intra-articulaire et l'injection de placebo dans les désordres internes temporomandibulaires (Moldez et al. 2018). En se focalisant plus particulièrement sur les arthropathies dégénératives, d'autres méta-analyses (Liu et al., 2018 ; Liu et al., 2020) ont montré une efficacité des injections de corticoïdes sur la réduction des douleurs (Liu et al. 2018) et sur l'amélioration fonctionnelle (Liu et al. 2020). Toutefois, l'utilisation de corticoïdes injectables de façon répétée provoque un effet déminéralisant des surfaces osseuses articulaires.

Nous avons cherché, au sein de l'Unité Inserm U1008, à développer de nouvelles techniques mini-invasives dans la prise en charge de ces dysfonctions temporomandibulaires articulaires. Nous avons dans un premier temps travaillé sur le développement d'un modèle animal de dysfonctions temporomandibulaires articulaires (ostéoarthrite temporomandibulaire) et étudié des traitements injectables de référence (acide hyaluronique). Nous avons ensuite étudié l'utilisation intra-articulaire de toxine botulique A (incobotulinumtoxinA) comme traitement novateur de l'ostéoarthrite temporomandibulaire, puis nous avons développé et étudié un

hydrogel à base de Chitosan et polymères de Cyclodextrine avec libération prolongée de substance pharmacologique active (*Projet MaxilloGel*). Cette thérapeutique novatrice permet d'inclure au sein d'une même substance injectable un effet de viscosupplémentation et une action anti-inflammatoire locale à libération prolongée limitant la prise d'AINS per os répétée et les effets systémiques de ces AINS.

# Partie I : Cohorte « Musculoskeletal Heritable Influences on Malocclusion, R21DE022427 »

## 3. La cohorte « Musculoskeletal Heritable Influences on Malocclusion, R21DE022427 »

## 4. Population

Il s'agit de patients adultes et adolescents ayant achevé leur croissance maxillo-faciale, et devant bénéficier d'une chirurgie orthognathique au sein du service de Chirurgie Maxillo-faciale et Stomatologie du CHRU de Lille afin de corriger leur dysmorphose dentofaciale.

## 5. Critères d'inclusion et de non inclusion

Étaient inclus les patients mineurs de plus de 12 ans ou majeurs devant bénéficier d'un traitement orthodontico-chirurgical pour la prise en charge d'une dysmorphose dentofaciale. La prise en charge chirurgicale devait comprendre au minimum une ostéotomie sagittale bilatérale de mandibule.
Étaient exclus les patients présentant une dysmorphose dentofaciale entrant dans un cadre syndromique, une pathologie inflammatoire systémique pouvant être responsable d'une ostéoarthrite temporomandibulaire, une pathologie neuromusculaire, et ceux présentant une denture de moins de 24 dents. Les femmes enceintes ou allaitantes étaient également exclues de cette étude.

## 6. Nombre de sujets nécessaires

L'objectif principal de la cohorte était de déterminer si le niveau d'expression des gènes d'intérêt est lié aux différents groupes cliniques de dysmorphoses dentofaciales (6 groupes). On ne disposait pas d'estimations sur les niveaux d'expression de ces gènes en fonction des groupes, permettant de calculer un nombre de sujets à inclure basé sur des hypothèses statistiques. Afin d'obtenir des estimations suffisamment précises, il convenait d'inclure au moins 30 patients dans chaque groupe. Nous avons proposé de recruter 40 patients dans chaque groupe, soit 240 patients au total, afin de tenir compte d'éventuelles données ininterprétables.

## 7. Autorisations et consentements

Ce travail est conforme aux principes éthiques de la déclaration d'Helsinki de 1964 révisée à Édimbourg en 2000 ; à la loi n° 2004-800 du 6 Août 2004 relative à la bioéthique ; à la loi de Santé Publique (loi n° 2004-800 du 9 Août 2004 relative à la politique de santé publique et loi n° 2006-450 du 18 avril 2006 pour la recherche) et à la loi n° 2004-801 du 6 Août 2004 relative à la protection des personnes physiques à l'égard des traitements de données à caractère personnel et modifiant la loi n° 78-17 du 6 janvier 1978 relative à l'informatique, aux fichiers, aux libertés.

Le protocole de recherche a été validé par l'Agence Nationale de Sécurité du Médicament et des Produits de Santé (ANSM) (Annexe 2), le Comité de Protection des Personnes « Nord-Ouest IV » (Certificat CPP12/44 ; Annexe 3), the *Temple University Institutional Review Board Committee* (Certificate 13438) (Annexe 4) et the *University of Pittsburgh Institutional Review Board Committee* (Certificate PRO12080373) (Annexe 5).

Au cours d'un entretien individualisé, les patients étaient informés des objectifs et du déroulement de l'étude et le consentement a été recherché par écrit. Le consentement des sujets mineurs de plus de 12 ans devait être recueilli par écrit même si le consentement du/des tuteur(s) légal(aux) était déjà obtenu.

Le travail a été financé par le *National Institute of Dental & Craniofacial Research* (grant to Dr. Sciote; *Musculoskeletal Heritable Influences on Malocclusion* - R21DE022427) dans le cadre d'un Consortium entre Lille, Pittsbrurgh et Philadelphie.

## 8. Données recueillies & collection biomédicale

Le recueil de données s'est étalé sur une période de 4 ans (Période d'inclusion de 3 ans / Participation de 1 an pour chaque patient). Son organisation et la collecte des échantillons biologiques sont schématisées dans la figure 1.

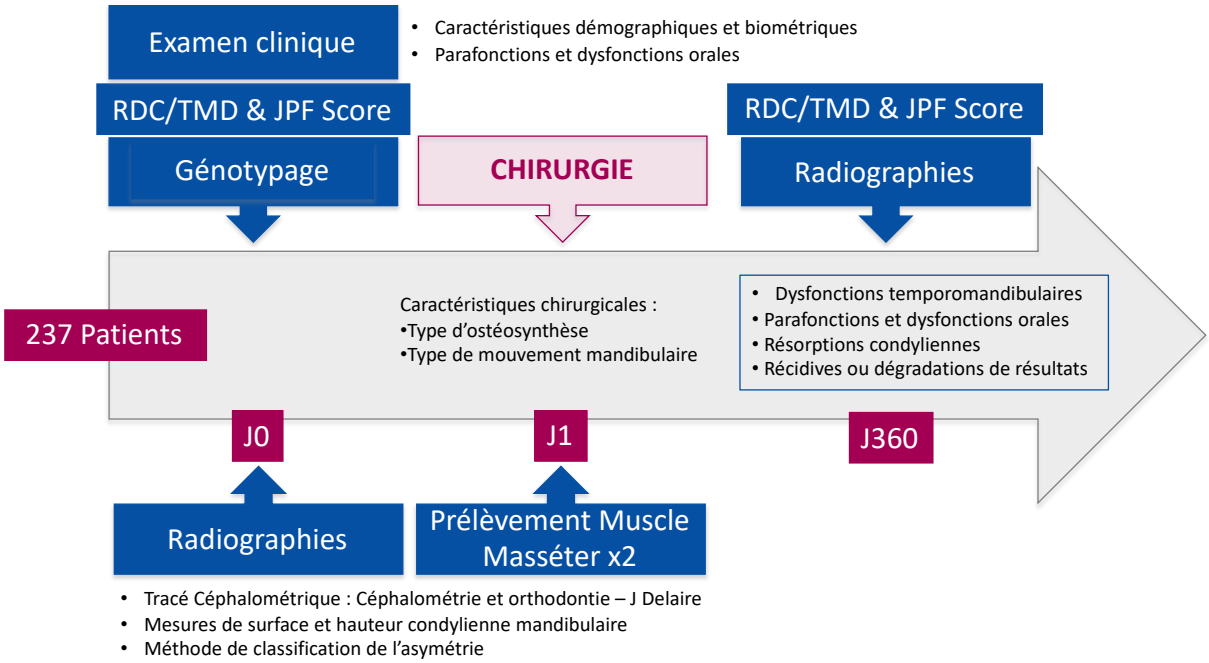


*Figure 1*

### 1. Données cliniques

*Données démographiques et biométriques*

Au cours d'un entretien individualisé étaient recueillis l'âge, le sexe masculin ou féminin, le type de profil orthognathique, les mesures biométriques faciales.

*Dysfonctions et parafonctions orales*

Les patients ont été invités à répondre aux questionnaires proposés par la « *Oral behavior Checklist* » tirée des RDC/TMD (au cours de la journée, vous mordez vous les doigts ? Vous rongez vous les ongles ? ou vous arrachez vous vos cuticules ?) (Ohrbach et al. 2008).

Une dysfonction ou dyspraxie orofaciale est définie comme une déviance constitutionnelle d'une des fonctions orofaciales que sont la déglutition, la parole, la respiration. Une rééducation fonctionnelle stricte est nécessaire pour corriger de telles habitudes (Ferri 2014). Les

dysfonctions recherchées dans cette cohorte étaient l'incompétence labiale, la protrusion/interposition linguale, la déglutition primaire, et la respiration buccale.

A contrario, les parafonctions orales sont des comportements, tics ou habitudes orofaciales, totalement indépendantes des fonctions précédemment citées. Ohrbach et Michelotti ont décrit les parafonctions comme des comportements buccaux, masticatoires, et faciaux qui ne remplissent aucun objectif fonctionnel (Ohrbach et Michelotti 2018). Cette définition très large peut inclure de nombreuses actions et habitudes pouvant être éradiquées sans rééducation. Nous avons recherché dans cette cohorte les parafonctions suivantes : bruxisme, onychophagie, succion du pouce ou de la tétine. Le bruxisme est la parafonction orale la plus fréquemment observée et étudiée. Dans un récent consensus international, Lobbezoo et al. ont défini le bruxisme comme une action répétitive des muscles de la mastication caractérisée par le serrage ou l'abrasion des dents et/ou par la propulsion ou l'exécution de mouvements latéraux de la mandibule, et ont précisé que son diagnostic pouvait être porté par l'interrogatoire et l'examen clinique (Lobbezoo et al. 2013, 2018). En revanche, nous n'avons pas différencié bruxisme du sommeil et bruxisme de l'éveil dans ce travail car l'élaboration du protocole d'étude a débuté avant la parution du consensus différentiant les rythmes circadiens du bruxisme.

*Dysfonctions temporomandibulaires*

Pour chaque patient étaient recherchée l'existence d'une dysfonction temporomandibulaire en préopératoire et 1 an après la prise en charger chirurgicale. Celle-ci était classée selon les critères pour le diagnostic des dysfonctions temporomandibulaires (DC/TMD) pour la clinique et la recherche, issus du *International RDC/TMD Consortium Network* et du *Orofacial Pain Special Interest Group* (Schiffman et al. 2014). Ce système de classification répond au modèle biopsychosocial de la douleur qui inclut un axe I correspondant à l'évaluation physique et un axe II évaluant le statut psychosocial et le handicap lié à la douleur. Les dysfonctions temporomandibulaires liées à la douleur les plus communes sont représentés par les myalgies, les arthralgies, et les céphalées attribuées aux dysfonctions temporomandibulaires ; les désordres internes les plus fréquents sont les déplacements du discaux réductibles et déplacement discaux réductibles avec blocages intermittents, les déplacements discaux non réductibles avec limitation d'ouverture buccale, les déplacements discaux non réductibles sans limitation d'ouverture buccale et les arthropathies dégénératives. L'utilisation des critères diagnostiques DC/TMD permet le diagnostic des myalgies avec une sensibilité de 90% et une spécificité de 99%, celui des arthralgies avec une sensibilité de 89% et une spécificité comprise de 98%, celui des déplacements discaux non réductibles avec limitation d'ouverture buccale

avec une sensibilité de 80% et une spécificité de 97% (Schiffman et al. 2014). En revanche, pour le diagnostic des autres désordres internes temporomandibulaires, la sensibilité et la spécificité de ce système de classification sont moins importantes.
La réponse au questionnaire « *Jaw Pain Function* » était recherchée. Ce questionnaire en 13 questions, d'abord proposé par Clark et al. (Clark et al. 1989) en 1989, permet de dépister les dysfonctions temporomandibulaires avec une sensibilité comprise entre 90.3% et 97.7% et une spécificité comprise entre 95.7% et 100% lorsque que le score (Score JPF) est supérieur ou égal à 6 (Gerstner et al. 1994). Undt et al. (Undt et al. 2006) ont validé en 2006 ce questionnaire dans une population germanique différente de la population initiale.

### 2. Données radiographiques

Des clichés télé-radiographiques de face, de profil, de vue inférieure et un panoramique dentaire étaient réalisés avant la prise en charge chirurgicale afin d'objectiver le type de dysmorphose dentofaciale. A partir de ce bilan radiographique, entièrement numérisé à l'aide d'un scanner vertical, plusieurs analyses étaient effectuées :

- Une classification de la dysmorphose était réalisée après mesures céphalométriques selon la méthode de Delaire et al. (Delaire et al. 1981). Les patients étaient ainsi classés selon les différentes composantes de leur dysmorphose dans les 3 sens de l'espace : sens sagittal (Classe II ou Classe III squelettique), sens vertical (*Open-bite* ou *Deep-bite*) et degré d'asymétrie.
- A partir de la radiographie panoramique dentaire étaient calculées des données relatives à la morphologie de chacun des deux condyles mandibulaires (Constant et al. 2017) (Figure 2). La ligne reliant le point le plus postérieur du condyle au point le plus postérieur de l'angle mandibulaire était d'abords tracée. Étaient ensuite définies la perpendiculaire à cette ligne passant par le point le plus bas de l'incisure mandibulaire et sa perpendiculaire passant par le point le plus élevé du condyle. La hauteur condylienne était mesurée entre ces deux perpendiculaires (Figure 2-A). La surface de l'unité condylienne était mesurée à l'aide d'un logiciel de mesure de surface (Mesurim®), du point le plus bas de l'incisure mandibulaire, à la *lingula mandibulae* puis perpendiculairement jusqu'au bord postérieur du *ramus* mandibulaire (Figure 2-B). Le modelage osseux était ensuite défini par un différentiel de hauteur et de surface entre les côtés droit et gauche d'un même patient.

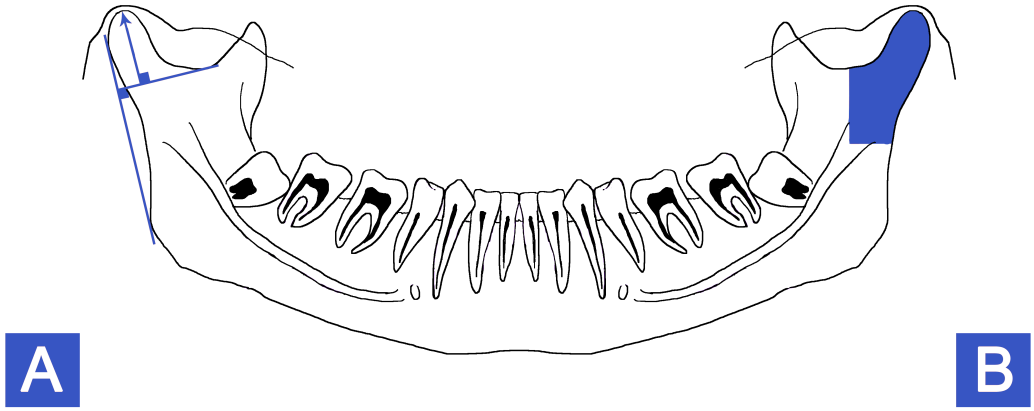


*Figure 2*

- L'asymétrie squelettique a été évaluée à l'aide du logiciel Dolphin® à partir de l'analyse des téléradiographies de face. Le système de classification de Baek et al. (Baek et al. 2012) a été utilisé pour classer l'asymétrie squelettique. Ce système de classification ayant été développé à partir d'images issues de Cone-Beam, nous avons adapté son analyse céphalométrique à partir des téléradiographies de face (Chung et al. 2017) (Figure 3). Six mesures céphalométriques ont été utilisées : l'inclinaison du plan occlusal, l'inclinaison maxillaire, la déviation du menton, la largeur mandibulaire au plan sagittal médian ou au menton, et la hauteur du ramus. Les patients étaient classés comme symétriques s'il n'y avait pas d'inclinaison maxillaire (<2◦), pas de déviation du menton (<2◦) et pas de différence significative de hauteur du ramus (<3 mm). Lorsque ils étaient considérés comme asymétriques, ils étaient classés dans l'un des quatre groupes suivants : Groupe 1 - latéralisation du corps mandibulaire uniquement (« asymétrie du corps mandibulaire »); Groupe 2 - différence de hauteur des ramus avec déviation du menton du côté ou le ramus est le plus court (« asymétrie des ramus »); Groupe 3 - différence de hauteur des ramus avec déviation du menton du côté ou le ramus est le plus long, contour des gonions plus proéminents sur le côté mandibulaire le plus large et inclinaison maxillaire inversée (« asymétrie atypique »); Groupe 4 - différence de hauteur des ramus avec déviation du menton du côté ou le ramus est le plus court et inclinaison maxillaire sévère (« asymétrie en forme de C »).

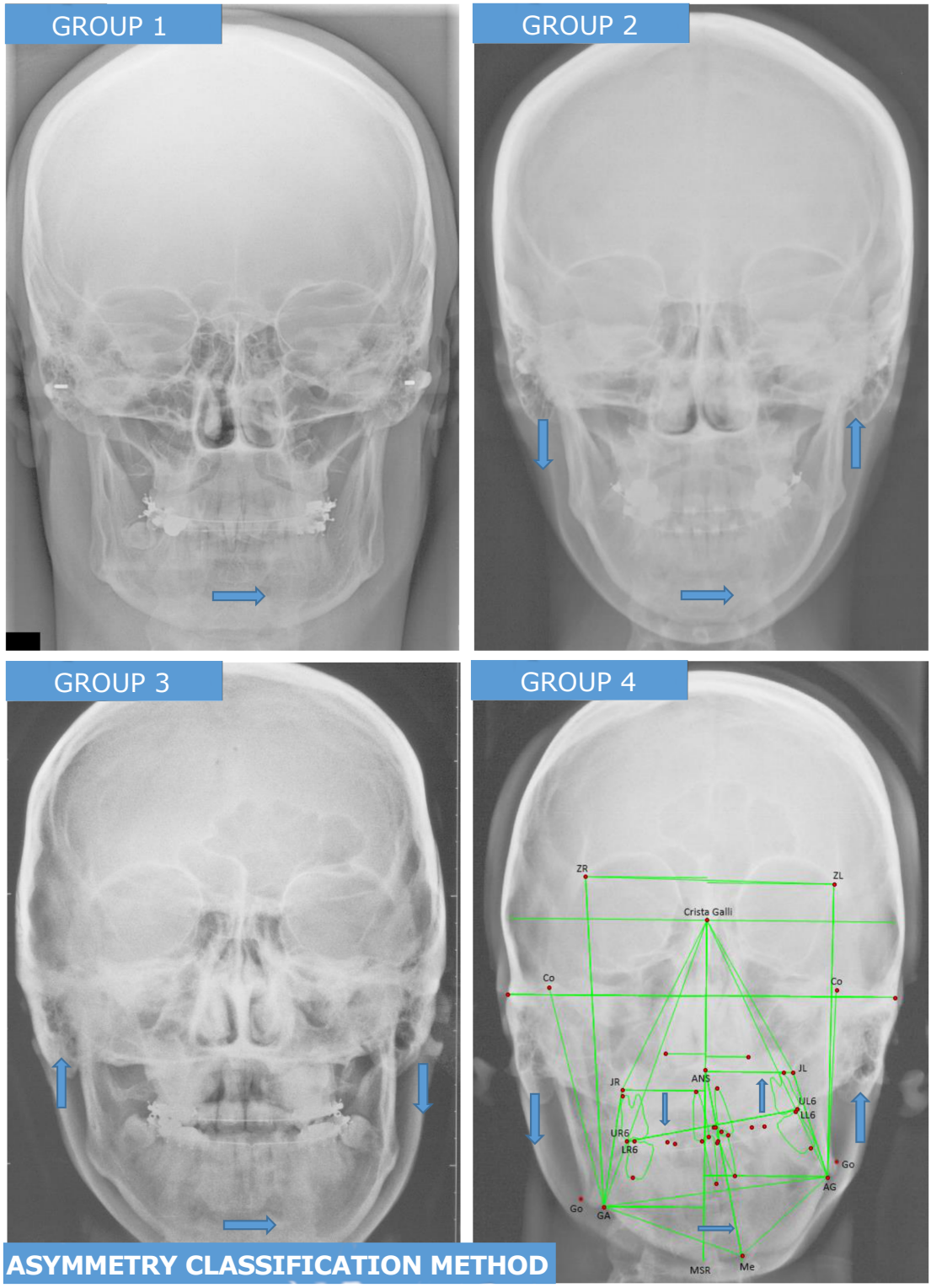
GROUP 1
GROUP 2
GROUP 3
GROUP 4
ASYMMETRY CLASSIFICATION METHOD
ZR
ZL
Crista Galli
Co
Co
JR
ANS
JL
UL6
LL6
UR6
LR6
Go
AG
Go
GA
MSR
Me

*Figure 3*

Le bilan téléradiographique était renouvelé à un an postopératoire dans d'évaluer la stabilité à long terme et en particulier afin d'étudier la morphologie condylienne (recherche d'une résorption condylienne postopératoire) (Ferri et al. 2016) ou de rechercher une dégradation de résultats/récidive (Ferri 2014).

### 3. Génotypage

Un prélèvement salivaire était réalisé en préopératoire à partir d'un kit spécifique Oragene® DNA (OG-250). L'analyse des échantillons a été réalisée par PCR quantitative en temps réel (TaqMan®) et analyse post-PCR (*end-point analysis*) avec un appareil de séquençage et de génotypage automatique à haut débit (ABI Prism 7900HT, Applied Biosystems, Foster City, CA), selon la méthode décrite par Zucchero et al. (Zucchero et al. 2004) sous la direction du Pr Vieira A.R.

Les polymorphismes nucléotidiques (*single nucleotide polymorphisms*) sélectionnés pour le génotypage étaient les suivants : rs1671064, rs1815739 and rs678397 pour le gène *ACTN3,* rs9373000, rs6569759, rs858339, et rs1409181 pour le gène *ENPP1*, rs1643821, rs3020318, rs3020377, rs2077647 pour le gène *ESR1*, rs1131611 pour le gène *PITX1* et rs2595110 pour le gène *PITX2.* Les polymorphismes nucléotidiques étudiés et leurs caractéristiques sont résumés dans le tableau 1.

*Tableau 1*

| Gène | Chromosome | SNP | Localisation | Variation allélique | Changement protéique | Type de SNP | 1000G MAF |
|---|---|---|---|---|---|---|---|
| *ACTN3* | 11 | rs1671064 | 66,560,202 | A | Arg523Gln | faux-sens | G = 0.4135 |
| | | rs1815739 | 66,560,624 | T | Arg577Ter | non sens | T = 0.4008 |
| | | rs678397 | 66,557,112 | C | | intron | T = 0.4203 |
| *ENPP1* | 6 | rs9373000 | 131,900,566 | G | | | G = 0.3594 |
| | | rs6569759 | 131,811,976 | G | | intron | A = 0.3880 |
| | | rs858339 | 131,832,757 | A | | intron | A = 0.2810 |
| | | rs1409181 | 131,828,160 | G | | intron | G = 0.4806 |
| *ESR1* | 6 | rs1643821 | 151,862,416 | A | | intron | A = 0.4852 |
| | | rs3020318 | 151,968,635 | C | | intron | C = 0.4105 |
| | | rs3020377 | 151,951,263 | A | | intron | A = 0.3804 |
| | | rs2077647 | 151,807,942 | C | Ser10= | synonyme | C = 0.4665 |
| *PITX1* | 5 | rs1131611 | 135,029,306 | T | Arg140= | synonyme | T = 0.1793 |
| *PITX2* | 4 | rs2595110 | 110,624,167 | G | | intron | G = 01516 |

### 4. Chirurgie orthognathique et prélèvement de muscle masséter

Chaque patient inclus devait au moins bénéficier d'une ostéotomie sagittale bilatérale de mandibule selon la technique d'Epker (Epker 1977). Cette ostéotomie sépare la branche montante de la mandibule de l'arche dentaire, permettant ainsi son repositionnement dans l'espace afin de corriger le trouble occlusal et la déformation morphologique (Figure 4).
Les mouvements chirurgicaux mandibulaires antéropostérieurs susceptibles d'influencer la position condylienne lors de la chirurgie orthognathique étaient colligés. On différenciait les avancées des reculs mandibulaires.

Le type d'ostéosynthèse était également recueilli. On distinguait les ostéosynthèses comprenant une ostéosynthèse antérieure « classique » par une ou deux plaques d'ostéosynthèse, des ostéosynthèses plus rigides comprenant en plus de l'ostéosynthèse antérieure une à deux vis bicorticales rétromolaires.

Au cours de la prise en charge chirurgicale étaient réalisés des prélèvements bilatéraux de muscles masséters, par la voie d'abords de l'ostéotomie mandibulaire d'Epker. Les échantillons étaient orientés puis cryoconservés selon la technique de Snap-freezing avant d'être acheminés au Centre de Ressources Biologiques de Lille où ils étaient anonymisés puis stockés, respectant les normes qualité ISO9001:2008 et NFS 96900, en attendant les analyses histomorphométriques.

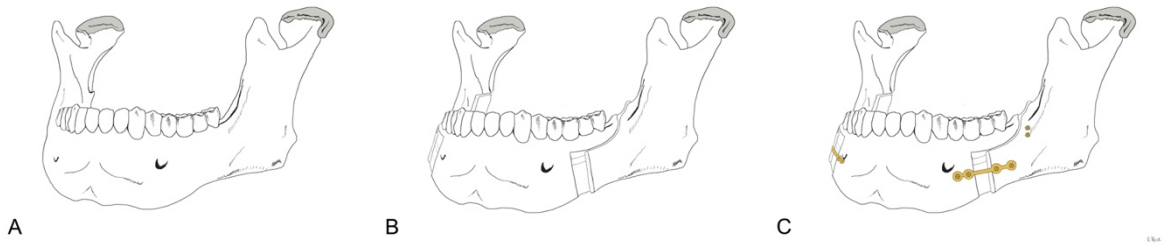


*Figure 4*

## 5. Histomorphométrie

Le muscle congelé a été cryosectionné à 10 µm d'épaisseur afin d'obtenir des coupes transversales sériées, et les coupes ont été montées sur des lames de verre pour l'immunocoloration avec des anticorps spécifiques des isoformes de la chaîne lourde de la myosine : anticorps anti MHC de type I (BA-F8), anticorps anti MHC de type II (BF-35), anticorps anti MCH de type IIA (SC-71), anticorps anti MCH Néonatale et anticorps anti MCH Atriale (Sciote et al. 1994; Sciote et Rowlerson 1998). Toutes les fibres dans les zones sélectionnées ont été classées par type et leurs sections transversales ont été mesurées avec le logiciel d 'analyse d'image Image J®. L'analyse histomorphométrique permettait de déterminer le type de fibres musculaires en croisant les réponses aux immunomarquages : fibres de type I, hybride (contenant à la fois des isoformes de MHC de type I et II), IIA, IIX, et de type néonatal et alpha cardiaque (Figure 5). L'analyse des coupes transversales permettait également de déterminer le nombre de fibres, leur surface moyenne et leur pourcentage d'occupation. La variabilité intra-individuelle dans la détermination de la surface de la fibre a été évaluée en répétant plusieurs fois le tracé morphométrique de toutes les surfaces des fibres au sein d'une même biopsie par le même examinateur.

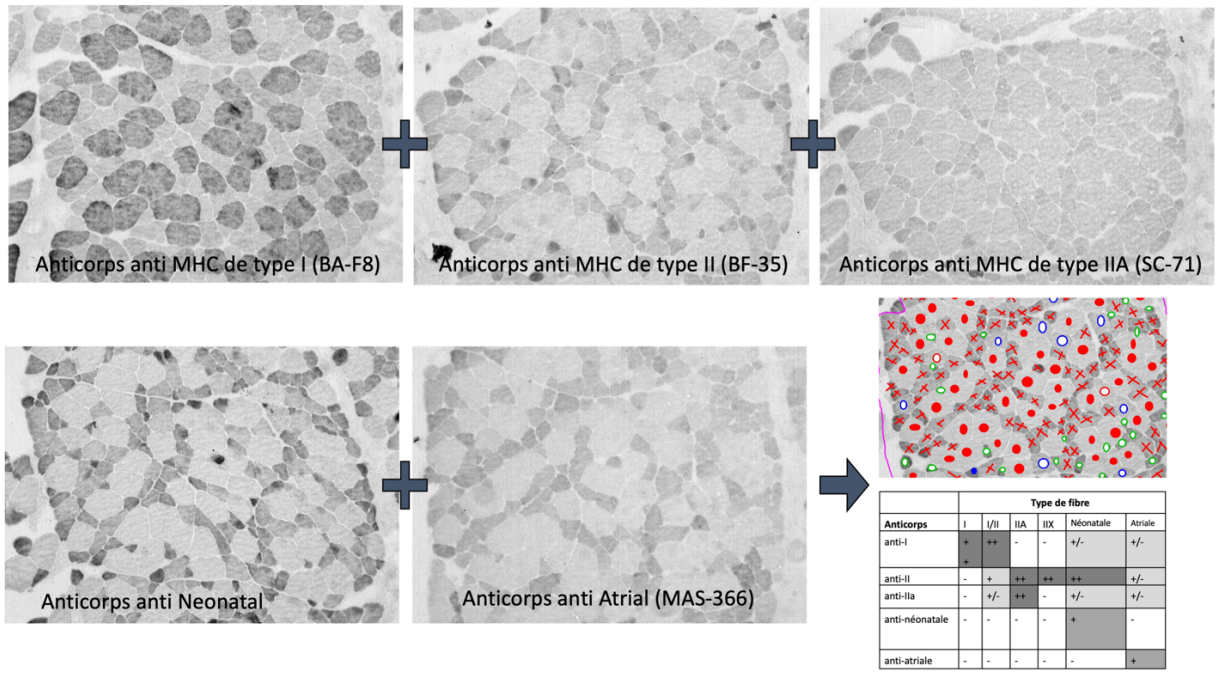


*Figure 5*

## Partie II : Rôle des gènes de la voie Nodale dans l'asymétrie faciale

INTRODUCTION : L'asymétrie faciale est une comorbidité morpho-fonctionnelle fréquente des patients présentant une dysmorphose dentofaciale. La connaissance de l'héritabilité des dysmorphoses dentofaciales progresse rapidement mais peu d'études se sont focalisées sur la composante asymétrique, dont l'imputabilité dysfonctionnelle n'est pourtant plus à démontrer. Le but de cette étude est d'identifier les différences d'expression de gènes clefs dans la genèse de la latéralité embryonnaire chez les patients présentant une asymétrie faciale par rapport aux patients symétriques au sein d'une cohorte de patients atteints de dysmorphoses dentofaciales.

METHODE : Des échantillons de muscle masséter bilatéraux ont été recueillis au cours d'une ostéotomie sagittale bilatérale de mandibule chez des patients présentant une dysmorphose dentofaciale. Les patients ont été classés selon la classe squelettique, la dimension verticale, et le caractère asymétrique ou non de leur dysmorphose dentofaciale. Une analyse de la différence d'expression des gènes a été réalisée sur les échantillons par la méthode des puces à ADN (Affymetrix HT2.O microarray global expression chips).

RESULTATS : L'expression globale des gènes était différente par analyse en composantes principales ($p<0.05$) chez les patients asymétriques par rapport aux patients symétriques et ceci quelle que soit la dysmorphose par ailleurs. Nous avons identifié des différences dans la voie de signalisation Nodale qui favorise le développement du mésoderme et de l'endoderme et la structuration de gauche à droite pendant l'embryogenèse. L'expression de *Nodal* et *Lefty* était 1.39 à 1.84 fois plus importante ($p<3.41x10-5$) chez les sujets asymétriques tandis que l'expression des modulateurs de la voie Nodale *Nomo 1, 2 et 3* était 5.63 à 5.81 ($p<3.05x10-4$) fois moins importantes. Les différences parmi les membres de la voie intracellulaire étaient de l'ordre de -7.02 à -2.47 (^<0.003). Enfin, *Pitx2*, un effecteur en amont de Nodal connu pour influencer la taille des fibres musculaires squelettiques de type II était aussi diminué chez les patients asymétriques mais de façon non significative ($p=0.06$).

CONCLUSION : Chez les patients présentant une dysmorphose dentofaciale avec asymétrie faciale, on constate une diminution de l'expression des gènes de la voie Nodale au sein du muscle masséter. Ces données suggèrent que la voie Nodale est régulée négativement afin de promouvoir le développement d'une asymétrie. La différence d'expression de *Pitx2* contribuerait également au développement musculosquelettique.

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# NODAL PATHWAY GENES ARE DOWNREGULATED IN FACIAL ASYMMETRY

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## Abstract

**Purpose—**Facial asymmetry is a common comorbid condition in patients with jaw deformation malocclusion. Heritability of malocclusion is advancing rapidly, but very little is known regarding genetic contributions to asymmetry. This study identifies differences in expression of key asymmetry-producing genes which are down regulated in facial asymmetry patients.

**Material and Methods—**Masseter muscle samples were collected during BSSO orthognathic surgery to correct skeletal-based malocclusion. Patients were classified as Class II or III and open or deep bite malocclusion with or without facial asymmetry. Muscle samples were analyzed for gene expression differences on Affymetrix HT2.0 microarray global expression chips.

**Results—**Overall gene expression was different for asymmetric patients compared to other malocclusion classifications by principal component analysis ($P<0.05$). We identified differences in the nodal signaling pathway (NSP) which promotes development of mesoderm and endoderm

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and left-right patterning during embryogenesis. *Nodal* and *Lefty* expression was 1.39–1.84 fold greater ($P<3.41\times10^{-5}$) whereas integral membrane *Nodal*-modulators *Nomo1,2,3* were −5.63 to −5.81 ($P<3.05\times10^{-4}$) less in asymmetry subjects. Fold differences among intracellular pathway members were negative in the range of −7.02 to −2.47 ($P<0.003$). Finally *Pitx2*, a upstream effector of *Nodal* known to influence the size of type II skeletal muscle fibers was also significantly decreased in facial asymmetry ($P<0.05$).

**Conclusions**—When facial asymmetry is part of skeletal malocclusion there are decreases of NSP genes in masseter muscle. This data suggests that the NSP is down regulated to help promote development of asymmetry. *Pitx2* expression differences also contributed to both skeletal and muscle development in this condition.

**Keywords**

ASYMMETRY; NODAL; PITX2; MALOCCLUSION

## Introduction

Bilateral asymmetry is an anthropometric trait commonly found in humans. For example, preference for right hand usage has been documented for over fifty centuries[1] and its resultant functional bias promotes asymmetry in arm bone length and diaphyseal breadth.[2] Differences in lower limb length of up to 10 mm exist in 95% of people, but are generally considered subclinical normal variations that may become more apparent later in life.[3] Asymmetry is also a common characteristic of the human face, but recognized subconsciously as a normal appearance.[4] Composites of left and right side mirror images of the human face, first studied by Hallervorden[5], each give a different appearance and are recognized as abnormal masked versions of a normally appearing asymmetric face.[6] Like the upper limbs, the right facial side is usually larger[6,7], but the chin is more often deviated to the left side.[7] The mandible along with constituent dental alveolar bone are typically the most symmetric given the ability of the dentition to adapt to differences by eruption and occlusal positioning.[8] Because of this adaptation, locations of the dental arches in the head and of the teeth within the dental arches are often slightly asymmetric, but not recognized clinically. Finally, the midpalatal raphe line of the palate is typically used as a reliable axis of symmetry, yet it usually differs with the maxillary arch center line by a few degrees.[6]

Craniofacial asymmetry requiring corrective surgery often results from specific pathological processes that ranges from childhood trauma[9], arthritis[10,11], condylar aplasia[12], and occular hypertelorism[13] to craniofacial anomalies such as unilateral craniosynostosis[14] and hemifacial microsomia.[15] More commonly, however, asymmetry is found in dentofacial deformity populations that have skeletal malocclusions. The National Dental Centre reports that among patients having orthodontic and surgical correction of malocclusion in the multiracial population of the Republic of Singapore, asymmetry occurs in 8% of skeletal class II and in 50% of skeletal class III individuals.[16] In contrast, the prevalence of asymmetry in skeletal class II is 28% and 40% for skeletal class III in a dentofacial deformities population undergoing surgical corrections at the University of North Carolina.[17] These data suggest that because asymmetry occurs through genetic and functional differences during growth and development, the proportion of sagittal, vertical

and transverse dysplasia present in a local population reflects their heritable background.[6] In a study of asymmetry that occurred with mandibular prognathism in two families for up to four generations, one family had a chin deviation to the left throughout the pedigree while the other had deviation almost always to the right.[18] This observation, along with more recent genetic findings, corroborates evidence that mandibular prognathism is a major transmissible Mendelian effect with dominant inheritance that has variable expressivity.[19,20] Nevertheless, the genetic influences that produce similarities of laterality in subsequent generations are yet to be fully appreciated.

Consistent measurement of laterality in subjects is fundamental to the study of facial asymmetry. One approach is to determine facial height differences that produce discordance between anatomic landmarks on both sides of the median sagittal plane.[21] Asymmetry detected by this approach may result from imbalanced growth and function of both the jaw [22] and associated muscles.[23,24] Obwegeser proposed that imbalances of mandibular growth occur in two different forms of prognathism; hemimandibular hyperplasia and hemimandibular elongation.[25] Hyperplasia produces a generalized enlargement to the affected side of the mandible and development of open bite, whereas elongation produces horizontal displacement of the mandible and chin towards the unaffected side with development of cross bite, rather than open bite. The way in which discrepancies of muscle function contribute to development of asymmetry are less clear.[26] It is known that muscle disuse produces decreased bone size and density, while increased physical activity have opposite effects, but the etiology of these relationships is not yet fully understood.[27] Bite force is associated with muscle cross sectional area[28] and mechanical advantage,[29] but muscle size is not always associated with facial skeletal form.[30–32] By contrast, despite the generation of small forces that are difficult to quantify, postural muscle tone to maintain the freeway space between the teeth and protect the airway appears to be more important.[33] Our approach has been to determine differences between fiber-type properties of the masseter muscle, which maintains mandibular posture in the conscious state. This work has demonstrated that variations in area and size of fiber types contribute to differences in the vertical dimension of facial growth. Bilateral comparisons of masseter muscle fiber types made in subjects with or without facial asymmetry show no differences with symmetry, but significant differences are found between sides when asymmetry is present. Irrespective of the type of skeletal malocclusion, type II fast-contracting fibers are increased in masseter muscle on the same side as the deviation[34] and the vertical facial dimension is decreased.[35,36]

*PITX2*, a gene active in the Nodal Pathway that determines left-right asymmetry during embryogenesis,[37,38] has recently been reported to be expressed in satellite cells of adult human skeletal muscle.[39] *PITX1*, another member of the *PITX* gene family, produces fiber atrophy when overexpressed in masseter muscle.[40] Collectively, these findings suggest that Nodal Pathway genes are active in adult skeletal muscle and may be key factors in development, growth and maintenance of facial asymmetry. In order to determine if symmetry pathways are active in adult humans we compared gene expression in masseter muscle between dentofacial deformity subjects with and without facial asymmetry.

## Materials and Methods

### SUBJECTS

Subjects were recruited from the dentofacial deformities population at the Université de Lille Department of Oral and Maxillofacial Surgery, undergoing orthodontic and maxillofacial surgery treatment for correction of malocclusion with marked jaw discrepancies. The subjects were recruited after they had signed an informed consent, and the research protocol was validated by the French Independent Ethical Committee (named CPP) and the Temple University IRB Committee. All subjects had at least a mandibular bilateral sagittal split osteotomy using Epker's technique. This osteotomy separates the ascending branch of the mandible from the dental arch, permitting repositioning in good occlusal position after adapted movement. Depending on the movement type and amplitude, it is sometimes necessary to cut the pterygo-masseteric sling to realign bones.[41] Finally a Tessier's distractor is used to completely separate the two bony pieces by more than one inch. During this procedure, the deep portion of the masseter muscle is exposed and muscle fibers are lacerated in the middle of the split. Before closing the surgical approach, to avoid muscle interposition between the bony pieces or being introduced in the suction drain, the lacerated muscle fibers are removed as clinical waste. These samples from left and right masseter are snap frozen in the operating room immediately after resection and then transported on dry ice to the Biological Resources Centre (BRC), Université de Lille, where they were they were managed and stored using procedures compliant with ISO 9001:2008 and the French Norm NFS 96 900 before express shipped to Temple University. Malocclusion classification is determined according to the type of surgical repositioning done to produce correction. Selected for study was either a left or right masseter muscle from 4 Class II deep bite malocclusion subjects, 4 Class II open bite and 3 Class III open bite. Facial asymmetry was present in one of the Class II open bites and one Class III open bite. In both asymmetry subjects masseter muscle from the side opposite of the chin deviation was used for genetic analysis. Finally one of the Class II open bite subjects had mandibular advancement surgery for correction of sleep apnea. This subject did not have facial asymmetry. Although genetic analysis was conducted on the sleep apnea subject, this patient was removed a priori from the comparisons in this study, and will be used to formulate a case report in a separate study. None of the subjects had systemic conditions, facial trauma, tumor, condylar hypertrophy, arthritis, or developmental conditions that might influence craniofacial growth.

### GENETIC ANALYSIS

Procedures for microarray target preparation and hybridization were done at the Molecular Profiling Facility of the University of Pennsylvania. Muscle samples were disrupted in QIAzol Lysis Reagent and RNA was isolated using a Qiagen miRNeasy Mini Kit according to the manufacturer's instructions. Quality of the total RNA was tested by Agilent Bioanalyzer and Nanodrop spectrophotometry. Protocols for microarray analysis were conducted as described in the Ambion WT Expression Manual and the Affymetrix GeneChip Expression Analysis Technical Manual. A 250 ng aliquot of total RNA was converted to first-strand cDNA by reverse transcriptase with poly(T) and random oligomer primers that incorporated the T7 promoter sequence. Second-strand cDNA synthesis was

followed by in vitro transcription with T7 RNA polymerase for linear amplification of each transcript. Resulting cRNA was converted to cDNA, fragmented, assessed by Bioanalyzer, and biotinylated by terminal transferase end labeling. Labeled cDNA (5.5$\mu$g) was added to an Affymetrix hybridization cocktail, heated at 99°C for 5 min and hybridized for 16 h at 45°C to Human Transcriptome 2.0 GeneChips (Affymetrix Inc., Santa Clara CA). After low (6X SSPE) and high (100 mM MES, 0.1M NaCl) stringency washes, the microarrays were stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A GeneChip 3000 7G scanner was used to collect fluorescence signal. Cel files containing individual probe intensities for each probe on each array were generated using Affymetrix Command Console software.

### STATISTICAL EVALUATION

Data Analysis of differentially expressed genes was analyzed with affymetrix® Transcriptome Analysis Console (TAC) Software. 11 cel files were imported to Partek Genomics Suite (v6.6, Partek Inc., St. Louis, MO) where RNA normalization was applied resulting in log2-transformed intensities for 70,534 transcript IDs corresponding to 24,953 unique gene symbols, their transcriptional variants and additional content provided on the platform. The one sleep apnea subject was removed prior to Principal Components Analysis (PCA) which determined the largest difference in global gene expression between the 10 remaining subjects. To find differentially expressed transcripts a t-test was performed comparing the 2 asymmetric samples (subjects number 1 and 2) to the 8 non-asymmetric samples. Resulting p-values were corrected for false discovery rate (FDR, step-up p-value) using the method of Benjamini and Hochberg as implemented in Partek Genomics Suite. This statistical approach is necessary to correct FDR (false discovery rate) since relatively small sample sizes are used to compare thousands of differences in gene expression levels. Fold-change was also calculated for each transcript. Differences were considered significant if step-up *p*-values were < .02 and fold differences were >±2 between groups. The present study utilizes the latest gene chip technology and analytical computations on a small subject population. No power analysis was included in the tests since accurate power analysis cannot be calculated without making too many unfounded assumptions.

Raw cel files for each of the 11 subjects were uploaded into the GEO database with the following ID link: _____ (*to be added when manuscript is accepted for publication*.)

## Results

### STASTICIAL DIFFERENCES BETWEEN GROUPS

Overall expression data for asymmetric patients sorted separate from subjects with other malocclusion classifications by PCA. The two subjects with facial asymmetry grouped as one cluster and all other malocclusion subjects, regardless of sagittal and vertical dimensions, grouped as a second cluster (Figure 1). Although there are genetic differences between the various malocclusion groups with symmetry, by far the largest genetic differences are when facial asymmetry is present or absent in skeletal malocclusions. Figure 1 shows a PCA plot summarizing the global inter-sample differences by visualizing high

dimensional variation in three dimensions. Each axis is a linear combination of all 70,534 transcripts, each with its own coefficient chosen in such a way to best show sample variations.

Transcripts were evaluated for differential expression and considered significant if they showed greater than ± 2 fold difference in gene expression and a step-up p-value < 0.05. Applying this reasonable cut off value between groups, approximately 22,000 gene transcripts were found to be significantly different, representing about 1/3 of the entire array. This reflects statistical cutoffs for significantly differentially expressed transcripts and is not related to results found from the PCA comparisons.

In order to better understand the biological relevance of the results, we evaluated a subset of genes which contribute to the normal formation of asymmetry. This gene set included Nodal morphogens which pattern the left – right axis in vertebrate development,[38] and Pitx2 which patterns muscle and craniofacial development.[42]

## CLINICAL PRESENTATION OF ASYMMETRY AND SURGICAL CORRECTION

Diagnosis was performed by clinical examination, evaluation of dental study models, panoramic radiographs and radiographic analysis of lateral, posterior-anterior, and submentovertex cephalograms. Surgical treatment planning was conducted using a computer-assisted cephalometric analysis from Delaire (Tridim and Orqual ceph, Orthalis).

On the posterior-anterior cephalometirc radiograph, asymmetry was identified using as vertical reference, the line passing through the crista galli and the superior part of the nasal septum, drawn perpendicular to the line between the intersections of the greater wings of the sphenoid bone and the lateral margins of the orbits. On the submentovertex cephalogram, asymmetry was evaluated for imbalances between the midpalatal raphe line (which represented the median sagittal plane) drawn perpendicular to the intercondylar axis reference line. Patients were included in the asymmetric group if the sagittal axis of the vomer bone was deviated relative to the median sagittal plane on the submentovertex radiograph. Mandibular asymmetry was identified by measuring the deviation of mandibular dental midline relative to the vertical reference line on the posterior-anterior cephalogram.

Subject number 1 presented a Class III open bite malocclusion, whereas subject 2 belonged to the Class II open bite group (Figures 2 and 3). From a facial asymmetry point of view, these two subjects had a marked posterior facial asymmetry. It included a significant deviation of the vomer bone and asymmetry of the pterygoid process of the sphenoid bone which could be clearly identified on the submentovertex and lateral cephalograms. The piriform apertures were also asymmetric and a tilt of the maxillary and palatine planes was also evident on the posterior-anterior radiographs. In addition to posterior facial asymmetry, subjects 1 and 2 presented a mild form of total facial asymmetry. Mandibular asymmetry included subject 1 with chin deviation to the right and subject 2 with chin deviation to the left.

The surgical correction for patient 1 combined a Le Fort I procedure to advance the maxilla with bilateral sagittal split osteotomy to retract the mandible and close the anterior bite, and

a reduction genioplasty. For patient 2, orthognathic surgery consisted of a Le Fort I osteotomy with maxillary impaction to correct the occlusal plane and anterior vertical excess, and a bilateral sagittal split osteotomy to advance the mandible. In both asymmetric subjects, bilateral sagittal split osteotomy performed a correction of mandibular asymmetry.

### DIFFERENCES IN NODAL PATHWAY GENE EXPRESSION

The normal formation of laterality in the heart and other organs is controlled by nodal pathway gene expression at specific embryonic developmental periods. Since the effects of asymmetric nodal pathway gene expression are well documented for first branchial arch structures, we investigated differences in nodal pathway gene expression *a priori* as potential candidate genes for facial asymmetry. There were very significant differences in gene expression (fold differences) for many of the Nodal pathway genes (Table 1). Table 1 reports both the individual gene expression values and averages for asymmetric subjects 1 and 2. For statistical comparisons, their average values were compared to average gene expression values for the 8 malocclusion subjects without facial asymmetry. Individualized data for subjects 1 and 2 is presented in Table 1 to demonstrate their close similarity in gene expression. 10 of the 22 Nodal pathway genes had significant expression levels for both fold differences and Step-up p-values (marked with an * in Table 1). This represented approximately 45% of the Nodal pathway genes of interest. Extracellular mediators *Nodal* and *Lefty* expression were 1.39–1.84 fold greater ($P < 3.41\times10^{-5}$) whereas integral membrane *Nodal*-modulators *Nomo1,2,3* were −5.63 to −5.81 ($P < 3.05\times10^{-4}$) less in asymmetry subjects. For the pathway's three surface Activin receptor molecules, two of the three were −2.47 (*ACTR2*) and −4.64 (*ACTR1A*) less in asymmetry subjects ($P < 5.06\times10^{-5}$ and $P < 9.65\times10^{-7}$). Four of the five positive intracellular pathway mediators had significantly negative fold differences in the range of −7.02 to −2.47 ($P < 0.003$). *PPM1A*, one of the two negative intracellular pathway mediators, had a significantly decreased fold difference of −3.79 with asymmetry ($P < 4.59\times10^{-6}$). *Pitx2*, an asymmetry factor known to influence the size of type II skeletal muscle fibers in adults was also decreased in facial asymmetry ($P < 0.06$), but with a fold difference less than 2.

Including the nodal pathway genes identified in Table 1, we were able to identify a total of 39 candidate genes of interest which have been reported to either participate in the nodal pathway or contribute individually to the development of asymmetry. In this larger grouping of genes only 28% reached our criteria for statistical significance, so caution must be used in interpreting our findings regarding nodal pathway genes in Table 1. However, the overall data trend for the genes listed in Table 1 was for moderate to large decreases in gene expression for 60% of them in the two subjects with facial asymmetry.

## Discussion

### DEVELOPMENT OF LEFT-RIGHT AXIS OF ASYMMETRY

During the third embryonic week formation of the three embryonic germ layers occurs through a process termed gastrulation. Gastrulation is initiated by formation of a primitive streak along the central axis of the epiblast through which invagination of epiblast cells form endoderm and mesoderm. At the cephalic end of the primitive streak the primitive node

forms an elevated area of cells which surrounds the primitive pit. Invading cells at the primitive pit migrate to the cephalic end of the embryo, forming the notochord. The notochord forms the midline axis of the embryo for development of the axial skeleton. The ectoderm overlying the notochord is induced to differentiate into the neural plate which initiates neurulation. Neurulation are the embryologic events that occur in the development of the nervous system. Neural folds develop as a thickening of cells to the left and right of the underlying notochord. A neural groove develops in the center of the folds that eventually unites at the upper aspects of the folds, forming the neural tube as the presumptive spinal cord. In the area where the neural folds are fusing, cells in the lateral crest area separate from the neuroectoderm and begin a migration passage into the developing branchial arches. These processes in late gastrulation and early neurulation periods begin as symmetric cellular patterns along the cephalic-caudal, dorsal-ventral and medial-lateral axes.[43,44]

In order for normal development to occur, this symmetry must be broken to develop clear differences in the left – right axis overtop the notochord. Symmetry breaking may occur through various intracellular changes in different species, but in mouse and other mammals there is conserved development of dynein molecular motors which rotate monocilia above the extracellular surface of the primitive node.[45] This introduces a lateral flow of morphogenic vesicles to the left side of the neural fold. Asymmetric cascades of gene expression from these morphogens leads to nodal expression in the left lateral plate mesoderm. Nodal is a transcription factor that initiates the nodal molecular pathway to induce chirality and asymmetry in endoderm and mesoderm germ layers during late gastrulation and neurulation.[46] There are three stages in development of the embryonic left – right axis: 1) A break in symmetry by directional flow from monocilia above the primitive node; 2) Formation of asymmetric gene expression first induced by the effects of nodal expression in left lateral plate mesoderm; and 3) changes in gene expression, through gradient density signaling, to pattern asymmetry into developing tissues and organs.[47] This third stage of organogenesis is established by the nodal downstream effector, *Pitx2*. This highly conserved transcription factor patterns asymmetry for correct *situs* positioning, gut and body rotations, and other lateral plate mesoderm derivatives.[48]

*Pitx2* is especially important to development of mesoderm-derived first branchial arch structures. In addition to lateral plate mesoderm, *Pitx2* is also expressed in cephalic and first branchial arch mesoderm during gastrulation.[49] In the first arch *Pitx2* is necessary to establish premyoblast specification of mesoderm and induction of ectoderm for tooth bud formation.[50] Masticatory muscles are absent in *Pitx2* mutant mice, while muscles in the other branchial arches develop, but have some significant deformations. Lack of skeletal structure development also occurs, but is limited to the first arch. This suggests that normal *Pitx2* expression is essential for induction of jaw development. Abnormal *Pitx2* signaling is well established before migration of neural crest cells into the arch, which disrupts normal organogenesis of the jaws.[42] After organogenesis is completed *Pitx2* continues to be expressed in most muscles of the head and trunk, and persists into adulthood.[50] It is especially important in maintenance of adult heart and extraocluar muscle functions. In heart formation, abnormal *Pitx2* function may result in aortic and septal defects.[51] In adult mouse and human heart *Pitx2* is expressed at much higher levels in the right atrium, in comparison

to the left atrium and ventricles. Mice with altered expression levels may develop atrial fibrillation. In humans, genetic variations close the *Pitx2* gene locus are associated with susceptibility to atrial fibrillation.[52] Similar experiments demonstrate that *Pitx2* expression is required for normal functioning of adult extraocular muscles.[53]

## NODAL PATHWAY GENE EXPRESSION IN ADULT MASSETER MUSCLE

We compared gene expression in masseter muscle between 10 subjects with jaw deformation malocclusion to determine the principle component differences in gene expression between groups (Figure 1). The two subjects with facial asymmetry clustered separately from the other malocclusion subjects even though one had a class II and the other a class III malocclusion. This allowed us to investigate the possibility that molecular pathways which pattern asymmetry in development could be active and regulated differentially in masseter muscle. Almost all of the molecular signals described in the Nodal Pathway,[38] were expressed at significantly higher or lower levels in the asymmetric subjects in comparison to the symmetric subjects (Table 1). Pathway genes were organized by gene ontology to determine how differential expression might contribute to development of facial asymmetry. *Nodal* was almost 2 fold increased in masseter muscle in the asymmetric subjects and *Nodal modulators* were almost 6 fold decreased indicating that *Nodal* overexpression was present with asymmetry. Nodal negative regulators *Lefty* 1 & 2 and *Cerberus* were elevated 1.5 to 2 fold with asymmetry which might indicate pathway activity to modulate Nodal overexpression. Most importantly however, *Pitx2* was down regulated, indicating that facial laterality might not have been adequately patterned. All of the positive and negative intracellular molecular mediators of the nodal pathway were down regulated, which might indicate that the intracellular pathway is not very active in masseter muscle from asymmetric subjects. The gene expression differences from microarray analysis present an exciting first insight into the relevance of Nodal Pathway genes in development of facial asymmetry, but must be interpreted with caution since 30% of total gene expression was different in subject comparisons. Therefore nodal pathway effects remain to be more fully explored in subsequent studies. Our results must be confirmed in additional RT-PCR experiments for individual gene expression levels, between left and right masseter muscle sides in symmetric and asymmetric faces, and for different types of facial asymmetry. Given our results, further exploration regarding gene expression differences between subjects with hemimandibular hyperplasia and hemimandibular elongation need further exploration.[25]

There is likely to be different expression levels for Nodal Pathway genes between left and right side masticatory muscles when asymmetry is present, since we know that fiber type distributions are significantly different between facial sides.[34] The Pitx family of genes may be particularly important influences of differences in masseter muscle fibers. *Pitx2* is required for initiation and progression of myogenic lineage of masticatory muscles,[42] and overexpression of *Pitx1* may cause skeletal muscle fiber atrophy.[40] Masseter muscle samples from the side opposite of the chin deviation had increased expression of *Pitx1* compared to muscle from symmetric subjects (Table 1). Fast-contracting type II fibers of masseter muscle on this facial side have decreased cell number and cell size, which could be an effect from increased Pitx1 expression. This relationship must be more fully explored in future studies. The fold differences for Pitx1 and 2 gene expression were less than two

between groups, and therefore not significant, but small variations in their gene levels may have biologically important effects given their key roles in development of first branchial arch structures.

Overall, nodal pathway gene expression may represent two distinct biological phenomena, remnants of an individual's developmental history and active differences in gene expression to maintain functional laterality. Differences in expression levels for the *nodal modula*tors (Table 1) most likely represent remnants of symmetry breaking from an earlier period. An intriguing possibility however, is that *Pitx1 and Pitx2* are actively expressed in masticatory muscle to maintain functional laterality when facial asymmetry is present. Future investigations will need to investigate differences in gene expression for these and similar candidate genes between left and right facial sides in relation to the pattern of skeletal asymmetry and masticatory muscle fiber type properties.

## UNIQUE FEATURES OF FACIAL ASYMMETRY IN OUR SUBJECTS

There are various kinds of craniofacial asymmetry and numerous methods of measuring the craniofacial skeleton.[8] Our study focuses on subjects with total facial asymmetry which included a significant posterior facial asymmetry. Among subjects from the facially symmetric group, some had a mild form of mandibular asymmetry but none had vertical asymmetry of the posterior face. So, the very clear distinction between the two groups suggests that genes known to contribute to asymmetry may lead to facial asymmetry, principally in the posterior areas of the face.

These findings are particularly encouraging given the role this posterior anatomic area plays in midfacial development, principally through growth of cranial base synchondroses. Indeed, cranial base synchondroses act as important growth centers for the craniofacial skeleton. Among midline chondral structures, the spheno-occipital synchondrosis seems to have the most prominent role in growth of the human skull.[54] Many authors have described the correlation between the cranial base angle and sagittal jaw dimesnions.[55–57] For example, an increased cranial base angle has been demonstrated to contribute to Class II malocclusion, whereas Class III deformity is associated with smaller linear and angular cranial base dimensions. Considering these factors, a bilateral asymmetry of posterior facial anatomy, including cranial base synchondroses and other posterior naris area sturctures, could lead to the development of mandibular asymmetry.

Moreover, the existence of a mild form of mandibular asymmetry in the group of symmetric patients suggests that posterior facial asymmetry with mild mandibular asymmetry and mandibular asymmetry which is isolated to the jaws are genetically distinct. Our subjects with posterior facial asymmetry clustered together, but subjects with isolated mandibular asymmetry did not cluster together from Principle Component Analysis. This highlights the multifactorial etiologies that may underlie different forms of mandibular asymmetry. To go further, we may be able to interpret the increase in the expression of *Pitx1* and the decrease in the expression of Pitx2 in masseter muscle as a mandibular compensatory mechanism to the asymmetry of the middle third of the face in subjects with posterior asymmetry. This compensatory mechanism could explain the mild form of mandibular asymmetry in these patients. Cranial base growth drives the face in a direction eventually compensated by the

growth of the jaw bones themself in conjunction with differences in jaw muscle function, given the fact that jaw muscle phenotype is related to facial asymmetry.[34]

In conclusion, this work helps us understand how sub classifications of facial asymmetry arise and how symmetry regulatory genes contribute to development of malocclusion. Further histologic analysis of masticatory muscle fiber types will be necessary to determine how variations in bilateral masticatory muscle phenotype contribute to jaw asymmetry in subjects with posterior facial asymmetry. Finally, the experimental approach used in this study should be explored in a larger subject population which includes a variety of different types of facial asymmetry.

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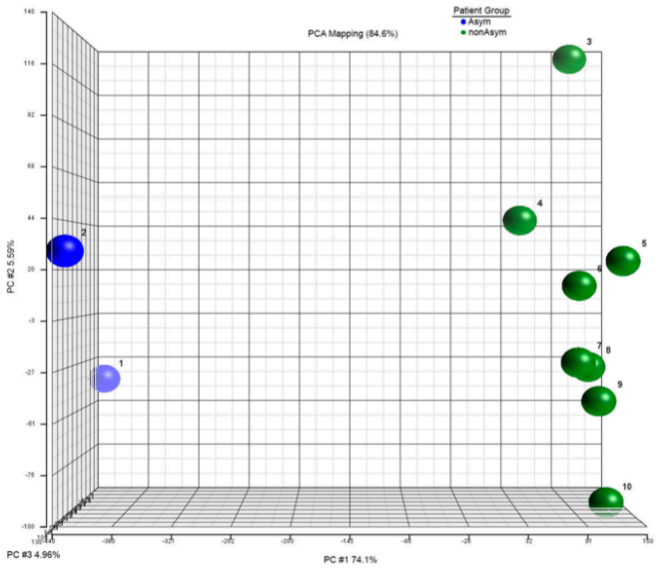


**Figure 1.**

Plot of differences in total gene expression in masseter muscle for 10 malocclusion subjects identified by Principal Components Analysis. Subjects 1 and 2 labeled in blue were a Class III open bite and Class II open bite respectively; both had significant posterior facial asymmetry with a mild form of total facial asymmetry. The remaining 8 malocclusion subject included 2 Class II open bites, 2 Class III open bites and 4 Class II deep bites. These subjects had facial symmetry and regardless of sagittal and vertical differences in malocclusion classification clustered as one group. Differences in expression of approximately 22,000 gene transcripts were highly significant when compared between the asymmetry pair and the remaining 8 malocclusion subjects.

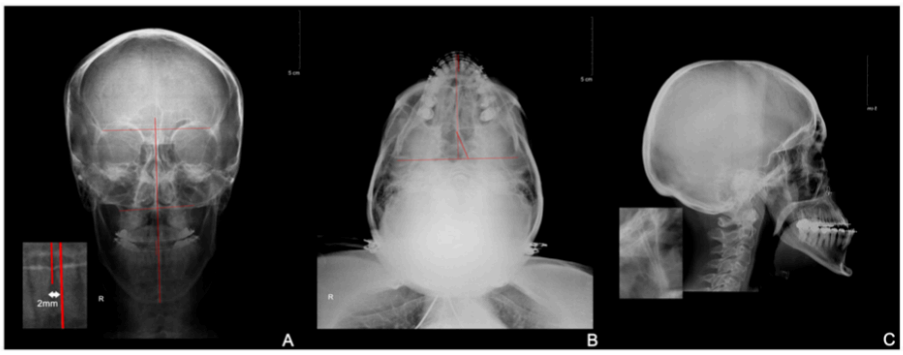


**Figure 2.**

A. Posterior-anterior cephalometric radiograph of subject 1 with total facial asymmetry. There was significant asymmetry of the middle third of the face, an asymmetry of the piriform apertures and tilt of palatal plane associated with a mandibular deviation on the opposite side of mandibular midline. B. Submentovertex cephalogram demonstrating significant deviation in the sagittal plane of the vomer bone relative to the median sagittal plane. C. Lateral cephalogram highlighting the duplication (asymmetry) of the left and right pterygoid processes of sphenoid bone.

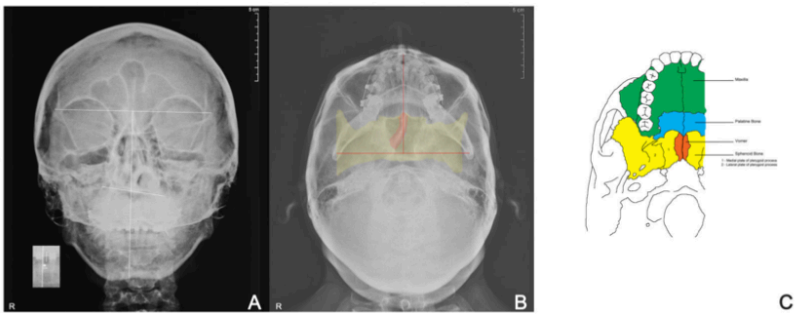


**Figure 3.**

Triptych illustration representing the posterior facial asymmetry found the vomer bone and the pterygoid process of the sphenoid bone in subject 2. A. Posterior-anterior cephalometric radiograph of subject 2 with total facial asymmetry. Asymmetry of the middle third of the face, asymmetry of the piriform apetures and tilt of palatal plane associated with a mandibular deviation on the opposite side of the mandibular midline were represented. B. Submentovertex cephalogram including midpalatal raphe line and intercondylar axis. Vomer bone is highlighted in orange whereas sphenoid bone is represented in yellow. C. Anatomical diagram representing the inferior view of cranial base.

**Table 1**

DIFFERENCES IN NODAL PATHWAY GENE EXPRESSION IN MASSETER MUSCLE FROM SUBJECTS WITH MALOCCLUSION AND FACIAL ASYMMETRY

| | | Expression Values* | | | | | |
|---|---|---|---|---|---|---|---|
| | | Asym (n = 2) | | | Sym (n = 8) | Asym vs Sym | |
| Genes | Transcript ID | Subject 1 | Subject 2 | mean | mean | Fold Diff | *P* Value |
| ***NODAL and NODAL* Modulators:** | | | | | | | |
| *NODAL* | TC10001365.hg.1 | 65.23 | 73.86 | 69.55 | 37.83 | 1.84 | 9E-06 |
| *NOMO1*** | TC16000180.hg.1 | 131.72 | 112.63 | 122.18 | 717.65 | −5.63 | 0.0003 |
| *NOMO2*** | TC16000912.hg.1 | 127.44 | 108.63 | 118.04 | 715.56 | −5.81 | 0.0003 |
| *NOMO3*** | TC16000194.hg.1 | 130.96 | 111.98 | 121.47 | 716.18 | −5.65 | 0.0003 |
| ***LEFTY* Negative Regulation of *NODAL*:** | | | | | | | |
| *LEFTY1* | TC01006387.hg.1 | 170.94 | 168.60 | 169.77 | 109.97 | 1.54 | 4E-06 |
| *LEFTY2* | TC01003895.hg.1 | 224.07 | 196.47 | 210.27 | 151.76 | 1.38 | 0.00003 |
| **Cerberus Negative Regulation of *NODAL*:** | | | | | | | |
| *CER1* | TC09000919.hg.1 | 50.74 | 85.77 | 68.26 | 34.72 | 1.90 | 0.0009 |
| **Downstream activator of *NODAL* Organogenesis Signaling:** | | | | | | | |
| *PITX1* | TC05001797.hg.1 | 189.97 | 176.86 | 183.42 | 162.14 | 1.13 | 0.02 |
| *PITX2* | TC04001471.hg.1 | 103.67 | 98.04 | 100.85 | 111.53 | −1.10 | 0.06 |
| ***Nodal* Cell Surface Receptors:** | | | | | | | |
| *ACTR1A*** | TC10001623.hg.1 | 55.38 | 52.97 | 54.18 | 252.63 | −4.64 | 1E-06 |
| *ACTR1B* | TC02002121.hg.1 | 201.56 | 209.44 | 205.50 | 215.37 | −1.05 | 0.29 |
| *ACTR2*** | TC02000378.hg.1 | 70.65 | 79.21 | 74.93 | 186.34 | −2.47 | 0.00005 |
| **Mediates *Lefty* Cell Surface Signaling:** | | | | | | | |
| *CFC1B* | TC02002344.hg.1 | 57.15 | 47.01 | 52.08 | 39.44 | 1.31 | 0.0005 |
| *CFC1B* | TC02000813.hg.1 | 52.27 | 45.91 | 49.09 | 38.57 | 1.27 | 0.0001 |
| **Positive Intracellular Mediators of the *NODAL* Pathway:** | | | | | | | |
| *SMAD1* | TC04000711.hg.1 | 117.73 | 109.43 | 113.58 | 139.64 | −1.22 | 0.094 |

| Genes | Transcript ID | Expression Values* | | | | Asym vs Sym | |
|---|---|---|---|---|---|---|---|
| | | Asym (n = 2) | | Sym (n = 8) | | | |
| | | Subject 1 | Subject 2 | mean | mean | Fold Diff | *P* Value |
| *SMAD2*** | TC18000496.hg.1 | 121.14 | 136.63 | 128.88 | 319.62 | −2.47 | 0.00002 |
| *SMAD3*** | TC15000622.hg.1 | 99.33 | 82.58 | 90.96 | 289.32 | −3.14 | 0.0001 |
| *SMAD4*** | TC18000998.hg.1 | 111.184 | 109.18 | 110.18 | 656.66 | −5.91 | 1E-06 |
| *USP9X*** | TC0X000195.hg.1 | 56.44 | 61.56 | 58.60 | 416.22 | −7.03 | 4.00E-07 |
| **Negative Intracellular Mediators of the *NODAL* Pathway:** | | | | | | | |
| *PPM1A*** | TC14000362.hg.1 | 84.86 | 88.31 | 86.59 | 331.38 | −3.80 | 5E-06 |
| *RANBP3* | TC19001087.hg.1 | 151.15 | 133.07 | 142.11 | 170.92 | −1.20 | 0.089 |
| **Positive Nuclear Transcription Factors:** | | | | | | | |
| *FOXH1* | TC08001753.hg.1 | 45.84 | 44.50 | 45.42 | 35.34 | 1.29 | 0.0002 |

Abbreviations: Asym, asymmetric (Subject 1, Class III Open bite; Subject 2, Class II Open bite); Sym, symmetric (8 Subjects with skeletal malocclusion without asymmetry); Fold diff, fold difference between average expression in Asym vs. Sym.

*Expression values are fluorescence signals detected for probes on the microarray, corrected for non-specific binding.

**Significant for step-up *P*-value with fold differences >±2 between groups.

# Partie III : Rôle des génotypes d'*ENPP1* et d'*ESR1* sur la géométrie condylienne et sur la santé articulaire temporomandibulaire

## I. Influence des génotypes d'*ENPP1* et d'*ESR1* sur le santé articulaire temporomandibulaire avant et après traitement chirurgical des dysmorphoses dentofaciales

INTRODUCTION : La chirurgie orthognathique a des conséquences morpho-fonctionnelles en impliquant des modifications des articulations temporomandibulaires. Néanmoins, le bénéfice articulaire de cette chirurgie n'est pas constant. Parmi les facteurs prédictifs d'une mauvaise réponse articulaire, le sexe féminin, la classe II ou encore l'hyperdivergence ont récemment été identifiés. Aucun facteur génétique n'a cependant été évoqué. L'objectif de cette étude était d'identifier les polymorphismes nucléotidiques associés à la santé articulaire préopératoire et à la réponse symptomatique dysfonctionnelle après chirurgie orthognathique.

METHODE : 120 patients consécutifs présentant une dysmorphose dentofaciale ont été inclus dans cette étude prospective, chacun devant par la suite bénéficier d'une prise en charge orthodontico-chirurgicale. Un prélèvement salivaire à visée génétique était réalisé au moment de l'inclusion. La réponse à un questionnaire validé relatif aux douleurs et fonctions de l'appareil manducateur était recherchée en préopératoire et à un an postopératoire. Treize polymorphismes de gènes d'intérêts ont été testés. Leur association avec un score préopératoire ou postopératoire orientant vers une dysfonction temporomandibulaire et avec l'aggravation du score en postopératoire a été étudiée.

RESULTATS : Le polymorphisme rs858339 du gène *ENPP1* était associé au score préopératoire (p=0,014). Le génotype TT représentait un facteur protecteur face aux dysfonctions de l'appareil manducateur (OR=0,286, IC95%[0,111;0,735], p=0,008). Réciproquement, le génotype hétérozygote AT représentait un facteur de risque de dysfonction temporomandibulaire par rapport au reste de la population (OR=3,910, IC95%[1,526;10,021], p=0,003). Néanmoins, les faibles effectifs dans les groupes AA doivent nous faire relativiser ces valeurs de p concernant le rs858339.

Aucun polymorphisme n'était associé statistiquement à un score postopératoire significativement élevé. Le polymorphisme rs1643821 du gène *ESR1* était associé à l'aggravation du score postopératoire de façon statistiquement significative (p=0,041). Le génotype AA représentait un facteur de risque de développer une aggravation symptomatologique en postopératoire par rapport aux deux autres génotypes (OR=4,176, IC95%[1,198;14,566], p=0,028).

CONCLUSION : Le polymorphisme rs858339 du gène *ENPP1* était associé aux dysfonctions temporomandibulaires dans une population de sujets présentant une dysmorphose dentofaciale. Le polymorphisme rs1643821 du gène ESR1 était associé à l'aggravation dysfonctionnelle post chirurgicale.

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# *ENPP1* and *ESR1* genotypes influence temporomandibular disorders development and surgical-treatment response in dento-facial deformities

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## Abstract

Dentofacial deformities are dys-morpho-functional disorders involving the temporomandibular joints (TMJ). Many authors have report a TMJ improvement in dysfunctional subjects with malocclusion after orthodontic or combined orthodontic and surgical treatment particularly for the relief of pain. In particular, few studies have highlighted the demographic and clinical predictors of response to surgical treatment. To date, no genetic factor has yet been identified as a predictor of response to surgical treatment. The aim of this cohort study is therefore to identify single-nucleotide polymorphisms associated with postoperative temporomandibular disorders (TMD) or with TMJ symptoms after orthognathic surgery. Here, we found the AA genotype of SNP rs1643821 (ESR1 gene) as a risk factor for dysfunctional worsening after orthognathic surgery. In addition, we have identified TT genotype of SNP rs858339 (ENPP1 gene) as a protective factor against TMD in a population of patients with dentofacial deformities. Conversely, the

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heterozygous genotype AT was identified as a risk factor of TMD with respect to the rest of our population. All these elements are particularly important to bring new screening strategies and tailor future treatment.

**Keywords**

Orthognathic Surgery; Temporomandibular Joint Disorders; Chronic Pain; estrogen receptor alpha; ectonucleotide pyrophosphatase phosphodiesterase 1

## Introduction

Dentofacial deformities are anatomic distortions of the jaws, dentition and face which affect about 20% of the population. They can occur in conditions such as cleft lip and palate and other craniofacial anomalies, or can constitute normal anthropometric variation. Apart from their morphological and dental consequences, dentofacial deformities are dys-morpho-functional disorders involving the temporomandibular joints (TMJ). Subjects with dental malocclusion have a higher rate of temporomandibular disorders (TMD) compared to the population with normal occlusal relationships (Celic et al., 2002; Egermark et al., 2003; Miller et al., 2004; Thilander et al., 2002). This is especially true when increased vertical dimension is a component of malocclusion (Hwang et al., 2006; Nebbe et al., 1998).

Temporomandibular disorders are defined by the American Academy of Orofacial Pain (AAOP) as a group of musculoskeletal and neuromuscular disorders, which involve the masticatory musculature, the temporomandibular joints and associated structures (de Leeuw and Klasser, 2013). The contribution of occlusion in TMD has been debated for a long time (Pullinger et al., 1993). There appears to be a TMJ improvement in dysfunctional subjects with Class II malocclusion after orthodontic treatment (Henrikson et al., 2000; Mohlin et al., 2004). In subjects with more severe malocclusions requiring a combined orthodontic and surgical treatment plan, recent works have enriched the debate (Abrahamsson et al., 2013; Dujoncquoy et al., 2010). *Abrahamsson et al.* (Abrahamsson et al., 2013) have shown in a controlled study on patients with dentofacial deformities, a positive impact on TMJ health from combined orthodontic and surgical treatment, particularly for the relief of pain. Angle Class I occlusion thus seems beneficial to temporomandibular biomechanics, but few studies have highlighted the predictors of response to surgical treatment (Pusan Korea Pusan National University et al., 2015; Scolozzi et al., 2015; Valladares-Neto et al., 2014). Female sex, amount of mandibular advancement and degree counterclockwise rotation of the mandible appear to be the main factors influencing the TMJ health after bilateral sagittal split osteotomy (Valladares-Neto et al., 2014). Women with class II malocclusion and increased vertical dimension as indicated by a larger mandibular plane angle, in fact have less TMJ improvements and are more prone to postoperative condylar resorption.

The scientific advances in the field of genetics since 2005 have allowed identification of single-nucleotide polymorphisms (SNPs) associated with TMD (Diatchenko, 2004). This work has expanded to identify dozens of additional SNPs in the hope of identifying individuals at high-rick prior to onset of TMD and to devise new therapeutic approaches for these patients (Melis and Giosia, 2014). To date, no genetic factor has yet been identified as

a predictor of response to surgical treatment. The aim of this study is therefore to identify single-nucleotide polymorphisms associated with postoperative TMD or with TMJ symptoms after orthognathic surgery.

## Materials & Methods

Subjects undergoing orthodontic and maxillofacial surgery treatment for correction of malocclusion were recruited between February 2013 and February 2014 from the Department of Oral and Maxillofacial Surgery at the University of Lille in France. They were recruited after signing an informed consent, and the research protocol was validated by the French independent ethical committee and the Temple University and the University of Pittsburgh institutional review boards. None of the subjects had systemic conditions, facial trauma, tumor, condylar hypertrophy, arthritis, or developmental conditions that might influence TMJ disorder.

Age, sex, facial biometrics and TMJ symptoms, were listed during the preoperative examination. Cephalometric data and classification were obtained by Delaire et al. (Delaire et al., 1981) analysis method. In that method, the line C3 is the superior line of the cranial base, drawn from M (junction of the nasofrontal, maxillofrontal and maxillonasal sutures) to Clp (apex of the posterior clinoid process). The line CF1 is perpendicular to line C3 registered at point FM, which is the intersect point of C3 and the line continuing to the anterior lacrimal crest. The adapted C1F1 angle represents the cranial angle corrected from abacus. Me Angle is defined as the angle between the line C3 and segment M-Me. Np Angle is defined as the angle between the line C3 and M-Npc (Nasopalatine canal). Anterior facial height is measured from Na (Nasion) to Me (Menton). After cephalometric analysis, malocclusion classification was also confirmed based upon the sagittal and vertical jaw repositioning required to execute the surgical treatment plan. The subjects were classified into 1 of 6 craniofacial morphologic groups that included 1 variation of sagittal skeletal jaw malocclusion, either Class II or Class III, and 1 variation of vertical skeletal jaw malocclusion — open bite, deep bite, or normal bite — relationship.

Facial anterior asymmetry was identified by measuring the deviation of mandibular dental midline relative to the vertical reference line on the posterior-anterior view. Vertical reference line is line passing through the crista galli and the superior part of the nasal septum, drawn perpendicular to the line between the intersections of the greater wings of the sphenoid bone and the lateral margins of the orbits. Facial posterior asymmetry was identified as described previously (Nicot et al., 2014). Regarding the comparative analysis, asymmetry was considered when ≥ 2mm (no asymmetry vs asymmetry).

Saliva samples from all subjects were stored in Oragene kits (DNA Genotek, Ottawa, Ontario, Canada) and used for DNA extraction and posterior genotyping.

All subjects had at least a mandibular bilateral sagittal split osteotomy using Epker's technique (Epker, 1977). This osteotomy separates the ascending branch of the mandible from the dental arch, permitting repositioning in good occlusal position after adapted movement. Surgical mandibular anteroposterior movements influencing condylar position

during orthognathic surgery were noted. Mandibular advancements were distinguished from mandibular setbacks. The type of osteosynthesis was also noted due to its potential influence on TMJ. It choice was mainly operator-dependent. The type of osteosynthesis was either "classic" – involving one or two titanium mini plates connecting the osteotomy site (Figure 1. A) – or one of increased rigidity, which had additional retromolar bi-cortical screws fixating the segments behind the mini plates – termed posterior rigid osteosynthesis (Figure 1. B).

### Evaluation method of TMJ response to orthognathic surgery

TMJ response was evaluated using scores obtained from the "Jaw Pain Function" questionnaire (Clark et al., 1989; Gerstner et al., 1994; Undt et al., 2006) evaluated before and at 1 year after orthognathic surgery. The survey includes 8 questions pertaining to jaw function and 5 to jaw function, with each question rated from 0 to 4 depending on the intensity of symptoms. A total score is thus obtained and an upper threshold or equal to 6 has been validated in the literature for the detection of TMD with a sensitivity of between 90.3% and 97.7% and a specificity between 95.7% and 100% (Gerstner et al., 1994). We used the scores in two independent ways to determine the presence or change in TMD symptoms as follows:

1. We first considered the JPF score one year after orthognathic surgery in order to select patients with severe TMJ symptoms postoperatively. Using the validated threshold to one postoperative year, we obtained a qualitative variable in two groups. Patients with JPF score ≥6 having TMD whereas patients with a JPF score <6 were considered with no dysfunction.
2. We then generated a variable based on JPF score evolution. This allowed us to identify select patients who had worsening symptoms postoperatively. The variable was obtained by subtracting the postoperative score from the preoperative score. (JPF = JPF preoperative score - postoperative score)

Considering a threshold of −2, we obtained a qualitative variable in two groups. Patients with JPF $\Delta \leq -2$ represented the subpopulation worsening "significantly" while patients with JPF $\Delta > 2$ were classified in the subpopulation with no worsening.

SNPs in genes of interest were selected based upon our previous findings of their potential role in development of malocclusion and or TMD, as described previously (Cheung et al., 2009; Deeley et al., 2015; Godel et al., 2014; Goding et al., 2003; Hottenstein et al., 2015; Kang et al., 2007; Kim et al., 2010; Liu et al., 2014; Nicot et al., 2014; Ribeiro-Dasilva et al., 2009; Sciote et al., 2015; Terkeltaub, 2001; Zebrick et al., 2014). These genes and their SNP characteristics are summarized in Table 1. Thirteen SNPs were selected for genotyping on all subjects and tested to determine whether specific allelic variants are correlated with changes in postoperative score using TaqMan chemistry (Life Technologies, Carlsbad, CA, USA) and end-point analysis in an automatic sequence-detection instrument (ABI Prism 7900HT; Applied Biosystems, Foster City, CA, USA), as described previously (Zucchero et al., 2004).

The characteristics of the population were presented with the usual rules of descriptive statistics: frequencies and percentages for categorical variables; mean and standard deviation for quantitative variables. The association between Delta JPF or postoperative score and clinical or surgical characteristics of the population was assessed by Chi-2 test for categorical variables or Fisher exact test in the case of small numbers. Quantitative variables were analyzed using Student's t test. When the distribution of the variable did not follow a normal distribution, a nonparametric Wilcoxon test was performed. After ensuring compliance with the Hardy-Weinberg equilibrium, the association of different SNPs was also sought by chi-square or Fisher's exact tests. For each SNP, the analysis was performed by considering the three genotypes separately. In case of significance, analysis was completed to compare each genotype to the two others and odds ratios (OR) with 95% confidence intervals (95% C.I.) were calculated. The significance level was set at $p = 0.05$. The p-value was not given in the tables when the actual conditions were not met

## Results

Between February 2013 and February 2014, 120 patients were included in the study. A total of 101 patients completed the postoperative questionnaire, constituting our analysis population. Patient selection is represented by the flow chart (Figure 2).

Our analysis population included a larger proportion of women (66.3%) and of young people (25.5 ± 11.3 years). Patients mainly had Class II dentofacial dysmorphosis (68.3%), with Normal (39.6%) or Open bite (41.6%). The biometric characteristics of the population of analysis are listed in Table 2. One can also note that there were 26.7% of patients with TMD before the orthognathic surgery. Patients with TMD were classified according to the original Research Diagnostic Criteria for Temporomandibular Disorders (RDC/TMD) (Reiter et al., 2012; Schiffman et al., 2014). Regarding axis I, the most common pain-related temporomandibular disorders were distributed as follows : 19 patients (70.4%) presented local myalgia and 17 patients (63.0%) presented arthralgia. Concerning the most common intra-articular temporomandibular disorders, 14 patients (51.9%) presented a disc displacement with reduction and 6 (22.2%) patients presented a disc displacement with reduction with intermittent locking. There were no positive diagnosis to Axis II.

### I. COMPARISON OF THE TWO GROUPS BY POSTOPERATIVE SCORE

There was no significant difference between the two groups regarding age, sex or biometric characteristics of patients, suggesting that the two populations were similar (Table 3). We found however more rigid fixation by bi-cortical retromolar screw in the <6 JPF score group (78.1%) compared to the ≥6 JPF score group (57.9%), however the findings were not statistically significant (p=0.085).

No SNP was found to be statistically associated with postoperative score (Table 4).

### II. COMPARISON OF TWO GROUPS BY DELTA JPF SCORE

There were no significant differences by age, sex or biometric, clinical and surgical characteristics between the 2 groups for Delta Score, suggesting that the two populations were similar (Table 5).

Among the 13 SNPs studied, single-nucleotide polymorphism rs1643821 of ESR1 gene had a statistically significant association with postoperative JPF score worsening ($p = 0.041$). There was especially a significantly greater proportion of subjects with the AA genotype in the JPF $\leq -2\ \Delta$ group (postoperative symptomatic worsening (26.1%) as compared to JPF$> -2\ \Delta$ group (no postoperative symptomatic worsening) (7.8%) (Table 6). The AA genotype was a risk factor of developing a symptomatic worsening postoperatively as compared to the other two genotypes (OR = 4.176, 95% C.I. [1.198, 14.566], $p = 0.028$) (Table 7).

### III. COMPARISON OF TWO GROUPS BY PREOPERATIVE SCORE

There were significantly ($p = 0.015$) more women in ≥6 preoperative JPF score group (85.2%) than in the <6 group (59.5%). Patients were also significantly older in the preoperative ≥6 JPF score group (28.7 ± 10.3 years) as compared to <6 group (24.4 ± 11.5 years) ($p = 0.018$).

There was no difference between the biometrical characteristics of the 2 groups of patients, suggesting that the two populations were comparable at the dentofacial level. Finally, 37% of patients were bruxers in the ≥6 preoperative JPF score group against 14.9% in the <6 preoperative JPF score group ($p = 0.015$) (Table 8).

Of the 13 SNPs analyzed, only rs858339 (ENPP1 gene) was associated with preoperative score ($p = 0.014$) (Table 9). There were proportionately more patients with the TT genotype in the <6 preoperative JPF score group compared to the ≥6 preoperative JPF score group (preoperative TMD) and this significantly ($p = 0.008$).

In particular, the TT genotype was a protective factor against TMD (OR = 0.286, 95% C.I. [0.111, 0.735], $p = 0.008$). Conversely, the heterozygous genotype AT was a risk factor of TMD with respect to the rest of the population (OR = 3.910, 95% C.I. [1.526, 10.021], $p = 0.003$). Nevertheless, these p values have to be treated with some care considering the low number of subjects in AA group for rs858339 (Table 10).

## Discussion

### I. ROLE OF SNP rs1643821 (*ESR1 GENE)* IN SYMPTOMATIC WORSENING OF TMJ AFTER ORTHOGNATHIC SURGERY

The role of estrogen in the occurrence of TMD has been investigated for many years. The prevalence of this condition in young women has presumed role for sex hormones (estrogen and progesterone) in TMJ remodeling (Arnett et al., 1996a, 1996b; Warren and Fried, 2001). The intensity of the painful symptoms appears to be greater in women for many anatomical locations, including the temporomandibular joints (Fillingim et al., 2009). Furthermore, there is a variation among women in the intensity of TMD related pain in the menstrual cycle; The intensity of pain is most extensive for the particular estrogen deficiency phase (menstrual phase) or during the phase of rapid change in estrogen levels (ovulatory phase and premenstrual phase) (LeResche et al., 2003). Supplementation in postmenopausal women by hormone replacement therapy as well as contraceptives in reproductive age women are also associated with an increased risk of developing a TMD (Fillingim and Edwards, 2001; LeResche, 1997; LeResche et al., 2003).

The biological activity of estrogen is mediated by specific receptors. The estrogen receptor is a protein of the steroid receptors family. There are two forms of this receptor, α and β. The α receptor is in particular found in the intra-articular cartilage and osteocytes and plays a role of intracellular mediators regulator. In rats, the α receptor is found in synovial cells, articular disc stromal cells and chondrocytes of the TMJ. There is an intensity gradient of age in favor of young subjects suggesting that estrogen is involved in the TMJ physiology differently depending on the age of the subjects (Yamada et al., 2003). In humans, Abubaker et al. (Abubaker et al., 1993) have highlighted the presence of estrogen receptors at the temporomandibular joint disk in both the subjects of male and female. These receptors are also found to a greater proportion in women with TMD than in subjects without TMD but these results can be highlighted significantly because of too low numbers. These results obtained by immunohistochemistry have subsequently been confirmed by transcriptomics (Ushiyama et al., 1999), in order to identify the estrogen receptor α as a fundamental biological mediator in the temporomandibular pathophysiology.

Recent studies have highlighted a significant association between single-nucleotide polymorphisms of ESR1 and symptoms of TMD (Kim et al., 2010) or TMJ osteoarthritis in women (Kang et al., 2007; Liu et al., 2014; Ribeiro-Dasilva et al., 2009). The gene encoding the α estrogen receptor is located on the long arm of chromosome 6 (6q25). It includes 7 introns and 8 exons on 140 kilobases (Han et al., 2011). The SNP rs1643821 of ESR1 gene is an alternative version with a transition from an A into a G at the intron 2 and having no known functional consequence. However, SNPs located at non-coding regions (introns) can affect splicing, transcription factors or may affect the production of non-coding RNA (Hull et al., 2007; Kwan et al., 2008). Similarly an SNP of the gene encoding the growth hormone (GH, Growth Hormone) affecting an intron is associated with physiological changes in hormone secretion (Millar et al., 2010), the rs1643821 variant could be another example of polymorphism affecting a functional intron by generating a specific modulation of transcription pathways related to estrogen. However, how this variant of the ESR1 gene modifies the articular symptomatic response to orthognathic surgery remains unknown. The rs1643821 single-nucleotide polymorphism of ESR1 gene has previously been significantly associated with the risk of osteoporotic fracture (Wang et al., 2012). Considering this work, our results do not show an association between rs1643821 and postoperative TMD. However, there is a significant association between this polymorphism and symptomatic worsening ($p = 0.041$). Especially, the AA homozygote mutated genotype is found as an aggravating risk factor for postoperative joint symptoms compared to wild-type genotype and the heterozygous genotype (OR = 4.176, 95% C.I. [1.198, 14.566], $p = 0.028$). These data allow us to strengthen the role of genetic polymorphism of ESR1 in the TMJ symptoms (Kang et al., 2007; Kim et al., 2010; Liu et al., 2014; Ribeiro-Dasilva et al., 2009).

On the biological level, the role of estrogen via the α receptor is well documented. It is involved in the pathophysiology of TMD through the inflammatory response, the bone mineralization and the nervous system (Craft, 2007). Via their α and β receptors in the peripheral nervous system as central nervous system (Laflamme et al., 1998; McEwen and Alves, 1999; Shupnik, 2002), estrogens are involved in the inflammatory response process and in the transmission of pain (Craft, 2007; Smith, 2006). Concerning the inflammatory response, estrogen negatively regulates the production of interleukin 1 (IL1) (Polan et al.,

1988), interleukin 6 (IL6) (Pottratz et al., 1994) and tumor necrosis factor α (TNF) (Ralston et al., 1990). IL1 and IL6-β cytokines are found in the synovial membrane of the temporomandibular joint during inflammation (Kubota et al., 1998), whereas TNFa and IL1 produced by monocytes / macrophages will promote cartilage resorption, inhibit proteoglycan synthesis and play a proinflammatory role in most of temporomandibular structures (Pettipher et al., 1986; Saklatvala, 1986). Treatment with estradiol seems then to have a protective effect on TMJ through reducing the number of monocytes in the articular synovial, and through reducing the production of proinflammatory cytokines (Guan et al., 2005; Pacifici, 1996, 1998). In rats, estrogen deficiency during puberty, induced by ovariectomy, predispose to alterations of TMJ by changes in serum levels of calcitonin and parathyroid hormone (Yasuoka et al., 2000). On the other hand, femoral bone mineral density is decreased in ovariectomized rats (Pajot et al., 2003). Estradiol is also involved in nociception mechanisms in TMJ. Using rats as model to study the pain has allowed identifying differences in masticatory muscle activity induced by glutamate (control of pain intensity) due to sexual hormones. Increasing concentrations of glutamate (the main excitatory neurotransmitters of central nervous system) seems to heighten awareness of TMD to noxious chemical stimuli (Cairns et al., 2002). In masticatory muscles, the increase of glutamate concentration plays an important role in the genesis of "myofascial pain syndrome" through activation of N-methyl D-aspartate receptors (NMDAr) (Castrillon et al., 2012).

Estradiol increases dose-dependently the trigeminal afferent discharge induced by NMDA injection into the masseter or temporomandibular joint in ovariectomized rats (Dong et al., 2007). These sex differences are due in part to an increase in the expression of peripheral NMDA receptors by neurons in the dorsal root ganglion mediated by estrogen (McRoberts et al., 2007). From a practical point of view, the discovery of a genetic marker of symptomatic worsening after orthognathic surgery allows us to glimpse new therapeutic drug targets (Lötsch et al., 2013). In this case, temporomandibular nociceptive pathways mediated by estrogen highlight the potential role of ketamine, an inhibitor of glutamate at NMDA receptors, in the treatment of selected patients. This treatment, used in the form of intraarticular injection, appears to have a different efficiency depending on the forms of TMJ dysfunction (Ayesh et al., 2008; Castrillon et al., 2012). Nevertheless its targeted use after orthognathic surgery in patients with a mutated genotype AA of SNP rs1643821 of the ESR1 gene could be a therapeutic application to study, given the role of estrogen in "myofascial pain syndrom" through activation of NMDA receptors.

Many studies have demonstrated a higher prevalence of pain among women. There are notably higher prevalence of musculoskeletal pain, orofacial pain and TMD (Fillingim et al., 2009). There are also differences in postoperative pain by sex published in many surgical fields and in particular in joint surgery (Fillingim et al., 2009). *Rosseland et al.* (Rosseland and Stubhaug, 2004), for example, have found an incidence of significant pain (at least moderate) greater in women immediately after surgery (2H) of a knee arthroscopy. This difference is no longer found at 1 year postoperatively while there is a prevalence of the most important pain in the elderly over 50 years old. In addition, the reduction in quality of life associated with this pain is most important in women at 1 year postoperatively (Rosseland et al., 2008). While the prevalence of pain appears to be greater in women in

many areas, pharmacological response (Gear et al., 1999) and non-pharmacological response (Krogstad et al., 1996) to the pain are more important, in particular in the orofacial field. For example there is a significant decrease of pain in women treated with a conservative treatment of TMD, whereas men show no symptomatological improvement (Krogstad et al., 1996). For our part, we have not found symptomatological changes by age or sex but these results may be related to a lack of power of the study.

## II. CONTROVERSY OF VARIABLES TO BE EXPLAINED: POSTOPERATIVE JPF SCORE OR Δ JPF SCORE?

Determining the influence of genetic polymorphisms on the articular dysfunctional symptomatology requires an evaluation criterion adapted to the dysfunctional articular symptoms and to integrate such diverse settings as symptoms worsening or symptomatic severity.

While the diagnostic criteria of TMD become more complex in order to separate various dysfunctional categories grouped under the same nosological framework (Schiffman et al., 2014), we wanted to use a validated score in the detection of TMD, in the broadest sense of the term. (Clark et al., 1989; Gerstner et al., 1994; Undt et al., 2006). Choosing this quantitative score with a pathological threshold defined in the literature then allowed us to consider both cases for which there was "severe" symptoms (TMD → ≥6 Postoperative JPF Score) and cases for which there was a dysfunctional symptoms worsening (≤ −2 Δ JPF Score).

One of the key criticisms of the variable named "postoperative JPF score" is that a ≥6 postoperative JPF score includes two kinds of patients: those with a score <6 and that have evolved after orthognathic surgery to a TMD and those who already had a ≥ 6 score and have remained highly symptomatic. Conversely, a <6 postoperative JPF score includes dysfunctional patients who were preoperatively and which are no longer under the criteria of the questionnaire postoperatively, but also patients who were not dysfunctional preoperatively and who have not presented a symptomatic evolution. In this way this criterion considers patients who are broader dysfunctional postoperatively without assuming their original status. Using this evaluation criterion is therefore to determine the SNP representing a risk factor for becoming or remaining dysfunctional postoperatively. Furthermore, it does not allow inclusion of patients whose score is below 6 postoperatively, while they sometimes can show an important variation of the score. A patient with a preoperative score of 0 who develops a postoperative score of 5, is for example not considered dysfunctional while he presents a larger change than a patient, with a preoperative score of 5, who develops a postoperative score of 6.

Therefore, the variable named " Δ JPF score" takes on its full meaning to determine patients with symptomatological worsening without these patients belonging to the TMD population. The threshold selected was determined arbitrarily and corresponds to a 30% worsening of the threshold defined in the literature for the detection of TMD. The main drawback of this endpoint is not to consider the intensity of the symptoms. Indeed, this criterion doesn't allow us to distinguish a patient with a score moving from 0 to 2 of a patient with a score moving from 6 to 8, whereas these two patients have completely different symptoms.

A variable integrating both subjects with symptoms worsening and "severe" subjects would have to increase the power of the statistical test, but it would have suffered a less accurate interpretation because of encompassing a larger volume of patients too distinct from one other from a symptomatic point of view. The choice to use these two variables independently allows us to separate statistically these two categories of patients. Finally, JPF score does not distinguish between different subtypes of TMD as recommended by the International Association for Dental Research (IADR) and the International Association of Study of Pain (IASP) (Schiffman et al., 2014) or the American Academy of orofacial Pain (AAOP) (Reiter et al., 2012).

## III. LIMITED CONFUSION FACTORS?

Although the link between TMD and orthognathic surgery is a subject of increased interest in the literature, a small number of quality studies have examined the predictors of symptomatic articular response to this surgical treatment (Dicker et al., 2015; Pusan Korea Pusan National University et al., 2015; Scolozzi et al., 2015; Valladares-Neto et al., 2014).

However, some clinical and surgical elements have been identified. From a demographic point of view, young age and female sex are factors influencing post-surgical joint symptoms (Valladares-Neto et al., 2014). Valladares-Neto et al. (Valladares-Neto et al., 2014) have also shown the importance of mandibular advancement, the degree of counterclockwise rotation of the mandible and the rigidity of fixation technique could be factors influencing the TMJ health. Thus, young adult females with mandibular retrognathism and increased mandibular plane angle would present a smaller joint improvement after orthognathic surgery and would be more prone to postoperative condylar resorption. Conversely, Sang-Yong et al. (Pusan Korea Pusan National University et al., 2015) noted an improvement of dysfunctional symptomatology and a good postoperative condylar stability after bimaxillary surgery in dysfunctional subjects with class III. These data are supported by Scolozzi et al. (Scolozzi et al., 2015) pointing out that the coexistence of maxillary retrusion and mandibular excess was an improving factor. Conversely, bimaxillary surgery in the broadest sense of the term is found in their work as a factor of symptoms worsening, as well as the existence of a "TMJ clicking", a pain in the preoperative TMJ palpation or pain in the masseter palpation.

In our work, we did not find any significant difference between the two groups according to the ΔJPF score or the postoperative JPF score for demographic, biometric or clinical characteristics and particularly for Class II or open bite malocclusion. Surgically, we did not find any significant association between the type of mandibular movement and the postoperative symptomatic aggravation. These results accord to those proposed by Dicker et al. (Dicker et al., 2015), which showed no significant changes in masseter or medial pterygoid muscles vectors and axial condylar rotation after a mandibular advancement. Moreover, unlike Valladares-Neto et al. (Valladares-Neto et al., 2014) our results noted a trend towards significance of the type of osteosynthesis by postoperative JPF score ($p = 0.07$). In other words, there is less rigid osteosynthesis in the dysfunctional group suggesting that a rigid fixation by bi-cortical retromolar screws could be a protective factor of postoperative TMD. These results will be developed on a larger population to be confirmed. It must be remembered that this type of osteosynthesis called "rigid" leaves little potential

for adaptation to the mandibular condyle and is experience-dependent; yet most orthognathic surgeons perform a condylar manual "repositioning" after sagittal split osteotomy of mandibular advancement. Although the evidence leads us to believe that the best position is the one involving the minimal stress of TMJ, analysis of the literature has not identified significant association between condylar positioning and condylar resorption (Ueki et al., 2012).

Bruxism supposed to be a confounding factor in the analysis of literature (Fernandes et al., 2015; Fillingim et al., 2009; Khawaja et al., 2015; LeResche, 1997; Manfredini and Lobbezoo, 2010; Ohrbach et al., 2011; Slade et al., 2011), is not found as a factor influencing the postoperative joint dysfunction or symptomatic worsening in our population. However, we found a significant association between bruxism and preoperative TMD confirming data already published. These results suggest that this factor, whose role seems to be important in the genesis of TMD, does not have a decisive role in the evolution of postoperative pain.

The absence of a significantly different in biometric and clinical features between the two groups according to the two variables to be explained (ΔJPF score and postoperative JPF score) enables us to relativize the existence of confounding factor, limiting the need for multivariate analysis for these two variables. However, this study suffers from relatively low numbers weakening its statistical power. Therefore, some results showing a trend of significance (posterior rigid osteosynthesis) could become significant by increasing the analysis population.

## IV. FACTORS ASSOCIATED WITH TMD IN OUR POPULATION

The results of our study have demonstrated a significant relationship between age ($p = 0.018$), sex ($p = 0.015$), bruxism ($p = 0.015$) and TMD. Our results are consistent with the distribution of TMD found in the literature according to age or sex (Fillingim et al., 2009; LeResche, 1997; Ohrbach et al., 2011; Slade et al., 2011). We found more female patients (85.2%; $p = 0.015$) in the preoperative TMD group with a higher median age between the second and the fourth decade ($28.7 \pm 10$, 3 years; $p = 0.018$). Bruxism was also associated with the presence of TMD ($p = 0.015$) confirming the literature data (Fernandes et al., 2015; Fillingim et al., 2009; Khawaja et al., 2015; LeResche, 1997; Manfredini and Lobbezoo, 2010; Ohrbach et al., 2011; Slade et al., 2011).

We also found a significant association between rs858339 (ENPP1 gene) and TMD ($p = 0.014$). ENPP1 is a gene encoding for ectonucleotide pyrophosphatase / phosphodiesterase 1, a transmembrane ectoenzyme that hydrolyzes extracellular molecules having a phosphodiester or pyrophosphate bond. This rather ubiquitous protein, highly expressed in chondrocytes and osteoblasts, plays a fundamental role in the regulation of bone mineralization (Goding et al., 2003). It hydrolyzes the inorganic pyrophosphate (PPi), a leading inhibitor of calcification, which inhibits crystal growth of hydroxyapatite (Terkeltaub, 2001). The role of ectonucleotide pyrophosphatase / phosphodiesterase 1 in inhibiting calcification has been demonstrated in a mouse model of ttw phenotype / ttw (tiptoe walking), which carries a nonsense mutation in the ENPP1 gene (Okawa et al., 1998). The ENPP1 −/ − mice develop a phenotype of hypermineralisation, calcification of tendons

and ligaments. These knock-out mice constitute a model for the human disease that is responsible for ossification of the posterior longitudinal ligament (OPLL) (Okawa et al., 1998). The phenotypic characteristics of ENPP1 −/ − mice include marked alterations in mineralization of the long bones and calvaria, and the peri-spinal soft tissue and arterial calcification (Mackenzie et al., 2012). Some SNPs in the ENPP1 gene have also been implicated in susceptibility, severity and progression of post-surgical OPLL (He et al., 2013). ENPP1 was also studied in the Framingham cohort, which identify polymorphisms associated with bone mineral density or bone morpho-biometrics. The SNP rs1974201 of ENPP1 gene is associated with bone morphology in the Framingham cohort (Cheung et al., 2009) while the SNP rs6569759 is associated with class III malocclusion (Deeley et al., 2015) suggesting that ENPP1 has a key role in bone morpho-biometrics. How these SNPs alter bone metabolism to affect bone formation remains unknown.

In our study, there was a greater proportion of subjects with TT wild genotype of rs6569759 (ENPP1 gene) in non-worsened group suggesting that this polymorphism was a protective factor dysfunction of TMD (OR = 0.286, 95% C.I. [0.111; 0.735], p = 0.008). Conversely, the heterozygous mutated polymorphism was a risk factor (OR = 3.910, 95% C.I. [1.526, 10.021], p = 0.003). The weak theoretical expected numbers for homozygous mutant genotype does not allow us to reach a conclusion due to the impossibility of using relevant statistical analyzes. As polymorphisms associated with surgical progression of OPLL, the presence of a mutant allele and a fortiori the homozygous mutant genotype of rs6569759 may thus have a role in regulating the TMJ bone mineralization by altering its function. Other ENPP1 polymorphisms have already been associated with TMD (Sciote et al., 2015). Our results are relatively consistent with those previously published making rs6569759 a key polymorphism of TMD. The results should, however, be confirmed with larger numbers to highlight the role of risk factor of homozygous mutant genotype.

## V. RESEARCH PERSPECTIVE: Oppera COHORT AND POTENTIAL GENES OF INTEREST

Many other genes of interest could be potential factors influencing the dysfunctional response to orthognathic surgery, particularly genes of the Oppera cohort (Slade et al., 2013). Oppera is a prospective cohort of 3262 adult subjects (18–44 years old) volunteers, recruited in four US sites between May 2006 and November 2008. This is the largest cohort to date to study the etiopathogenic factors of TMD. 2737 subjects followed for 2.8 years, have completed questionnaires to identify the occurrence of 260 cases of TMD.

Among the various research areas within this cohort, a component concerned predictive genes of TMD. Thus, 3295 SNP representing 358 genes known to be involved in pain perception systems were studied. None SNPs has been identified as significantly associated with the occurrence of TMD. However, they were many SNPs correlated with intermediate phenotypes TMD (Slade et al., 2013; Smith et al., 2011, 2013). On the other hand, polymorphisms of COMT genes, ESR1 genes and GCH1 genes interact to influence the enzymatic activity of catechol-O-methyltransferase, musculoskeletal pain, and mood constituting a phenomenon of epistasis (Smith et al., 2014). The study of CACNA2D1, a gene of interest in the cohort Oppera encoding subunit alpha-2 / delta-1 of voltage dependent calcium channels implicated in neuropathic pain, has a differential expression in the

masseter muscles according and sex between women with or without TMD myalgia-type (Godel et al., 2014; Sciote et al., 2015). All of these genes constitute a gene pool of interest in the study of polymorphisms that may influence the dysfunctional worsening after orthognathic surgery.

## Conclusion

Although long debated, the combined orthodontic and surgical treatment of dentofacial dysmorphosis seems beneficial to temporomandibular symptoms. Several demographic, clinical or surgical features influencing the joint response were found in recent clinical studies. We have identified the AA genotype of SNP rs1643821 (ESR1 gene) as a risk factor for dysfunctional worsening after orthognathic surgery. In addition, we have identified TT genotype of SNP rs858339 (ENPP1 gene) as a protective factor against TMD in a population of patients with dentofacial deformities. Conversely, the heterozygous genotype AT was identified as a risk factor of TMD with respect to the rest of our population. All these elements are particularly important to bring new screening strategies and tailor future treatment. Results should, however, be completed on a larger cohort.

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**Perspective**

This study allows us to identify sub-populations at high risk of developing postoperative temporomandibular disorders after orthognathic surgery procedures. Many other genes of interest could be potential factors influencing the dysfunctional response to orthognathic surgery, particularly genes of the Opera cohort.

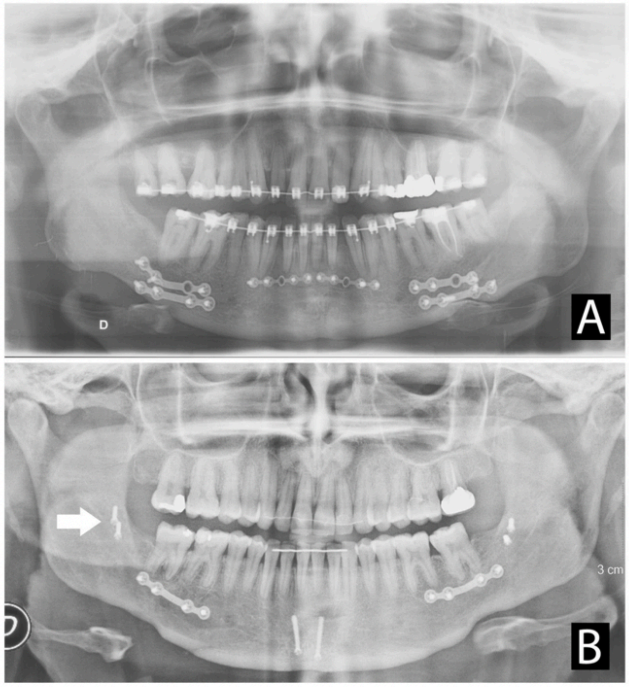


**Figure 1.**
Panoramic radiographs representing a "classic" osteosythesis (A) involving one or two titanium mini plates connecting the osteotomy site and one of increased rigidity (B) which had additional retromolar bi-cortical screws (white arrow) fixating the segments behind the mini plates. *Note that osteosynthesis technique is also different for genioplasty. It does not represent a confounding factor given the absence of biomechanical role on occlusion or TMJ.*

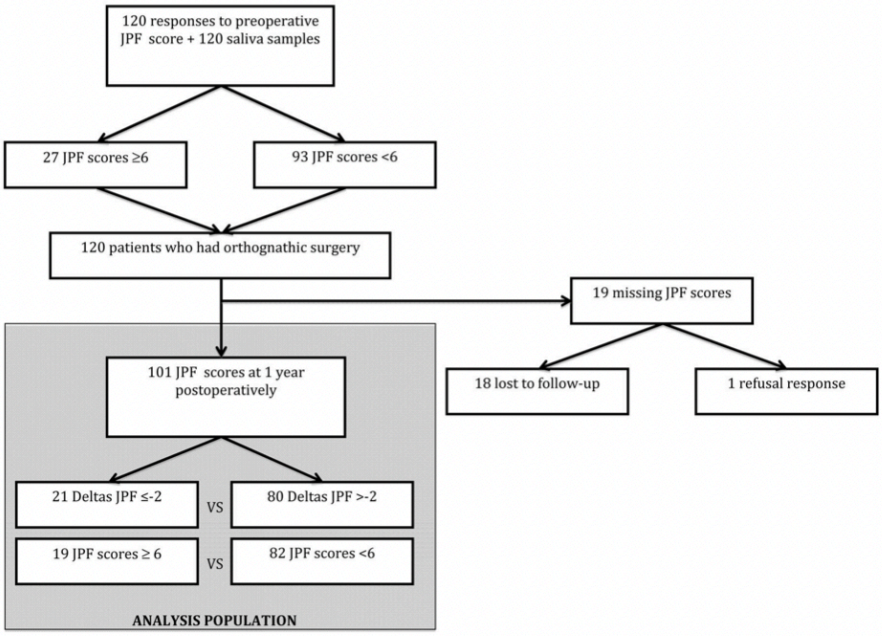


**Figure 2.**
Flow chart of patients

**Table 1**

Description of analyzed SNP (source: *NCBI variation viewer*)

| Gene | Chromosome | SNP | Location | Allelic variation | Protein change | SNP type | 1000 Genomes Minor Allele Frequency | Mechanism | References |
|---|---|---|---|---|---|---|---|---|---|
| *ACTN3* | 11 | rs1671064 | 66,560,202 | A | Arg523Gln | Missense | G = 0.4135 | - Muscle determinism | (25–27) |
| | | rs1815739 | 66,560,624 | T | Arg577Ter | Nonsense | T = 0.4008 | - Dentofacial deformities | |
| | | rs678397 | 66,557,112 | C | | Intron variant | T = 0.4203 | - TMD | |
| *ENPP1* | 6 | rs9373000 | 131,900,566 | G | | Intron variant | G = 0.3594 | - Bone mineralization | (27–31) |
| | | rs6569759 | 131,811,976 | G | | Intron variant | A = 0.3880 | - Dentofacial deformities | |
| | | rs858339 | 131,832,757 | A | | Intron variant | A = 0.2810 | - TMD | |
| | | rs1409181 | 131,828,160 | G | | | G = 0.4806 | | |
| *ESR1* | 6 | rs1643821 | 151,862,416 | A | Ser10= | Intron variant | A = 0.4852 | - TMD | (32–35) |
| | | rs3020318 | 151,968,635 | C | | Intron variant | C = 0.4105 | | |
| | | rs3020377 | 151,951,263 | A | | Intron variant | A = 0.3804 | | |
| | | rs2077647 | 151,807,942 | C | | Synonymous | C = 0.4665 | | |
| *PITX1* | 5 | rs1131611 | 135,029,306 | T | Arg140= | Synonymous | T = 0.1793 | - mandibular development | (20,36) |
| *PITX2* | 4 | rs2595110 | 110,624,167 | G | | Intron variant | G = 01516 | - Facial Asymmetry | |

**Table 2**

Description of analysis population

| Characteristics of analysis population | N=101 |
|---|---|
| **Age** *my (DS)* | 25.5 (11.3) |
| **Females** *n (%)* | 67 (66.3) |
| ***Biometrical characteristics:*** | |
| **Sagittal** *n (%)* | |
| - Class I | 1 (1) |
| - Class II | 69 (68.3) |
| - Class III | 31 (30.7) |
| **Vertical** *n (%)* | |
| - Normal Bite | 40 (39.6) |
| - Open Bite | 42 (41.6) |
| - Deep Bite | 19 (18.8) |
| **Asymmetry** *n (%)* | |
| - No asymmetry | 34 (33.6) |
| - Minor asymmetry | 23 (22.8) |
| - Asymmetry > 2 mm | 39 (38.6) |
| - Facial posterior asymmetry | 5 (5) |
| **Me Angle** (°) *my (DS)* | 85.4 (6.3) |
| **Np Angle** (°) *my (DS)* | 87.0 (4.5) |
| **Adapted C1F1** (°) *my (DS)* | 86.9 (3.0) |
| **Facial height** (Na-Me in mm) *my (DS)* | 121.3 (10.0) |
| **Preoperative JPF score ≥6** *n (%)* | 27 (26.7) |

N : number of observation; my : mean ; DS : standard deviation; n (%) : number of observation (percentage)

**Table 3**

Comparison of patients by postoperative JPF score: <6 postoperative JPF score group vs ≥6 postoperative JPF score group (postoperative TMD)

| | JPF score <6 N=82 | JPF score ≥6 N=19 | p |
|---|---|---|---|
| **Age** *my (DS)* | 26.1 (11.9) | 23.1 (7.8) | 0.734 |
| **Females** *n (%)* | 52 (63.4) | 15 (79.0) | 0.197 |
| ***Biometrical characteristics:*** | | | |
| **Sagittal** *n (%)* | | | |
| - Class II | 54 (65.9) | 16 (84.2) | 0.118 |
| - Class III | 28 (34.1) | 3 (15.8) | |
| **Vertical** *n (%)* | | | |
| - Normal Bite | 31 (37.8) | 9 (47.4) | 0.546 |
| - Open Bite | 34 (41.5) | 8 (42.1) | |
| - Deep Bite | 17 (20.7) | 2 (10.5) | |
| **Asymmetry** *n (%)* | | | |
| - No asymmetry | 27 (32.9) | 7 (36.8) | 0.745 |
| - Asymmetry | 55 (67.1) | 12 (63.2) | |
| **Me Angle** *my (DS)* | 85.6 (6.0) | 84.6 (7.3) | 0.350 |
| **Np Angle** *my (DS)* | 87.0 (4.3) | 86.9 (5.4) | 0.966 |
| **Adapted C1F1** *my (DS)* | 87.0 (3.0) | 86.8 (3.2) | 0.958 |
| **Facial Height** (mm) *my (DS)* | 121.9 (10.4) | 118.9 (8.2) | 0.258 |
| **Bruxism** *n (%)* | 16 (19.5) | 5 (26.3) | 0.536 |
| ***Surgical characteristics:*** | | | |
| **Type of osteosynthesis:** | | | |
| - Posterior rigid osteosynthesis | 64 (78.1) | 11 (57.9) | 0.085 |
| **Type of mandibular movement:** | | | |
| - Mandibular advancement | 58 (70.7) | 16 (84.2) | 0.232 |

N : number of observation; my : mean ; DS : standard deviation; n (%) : number of observation (percentage); p : p-value

**Table 4**

Comparison of SNP genotypes by postoperative JPF score: <6 postoperative JPF score group vs ≥6 postoperative JPF score group (postoperative TMD)

| Gene | SNP | N | Genotype | <6 postoperative JPF score | ≥6 postoperative JPF score | Groups | p |
|---|---|---|---|---|---|---|---|
| *ACTN3* | rs1671064 | 100 | AA n (%) | 15 (18.5) | 4 (21.1) | AA vs GA vs GG | 0.723 |
| | | | GA | 50 (61.7) | 10 (52.6) | | |
| | | | GG | 16 (19.8) | 5 (26.3) | | |
| | rs1815739 | 101 | CC | 17 (20.7) | 4 (21.1) | CC vs TC vs TT | 1 |
| | | | TC | 48 (58.6) | 11 (57.8) | | |
| | | | TT | 17 (20.7) | 4 (21.1) | | |
| | rs678397 | 95 | CC | 17 (22.1) | 4 (22.2) | CC vs CT vs TT | 0.939 |
| | | | CT | 42 (54.5) | 9 (50) | | |
| | | | TT | 18 (23.4) | 5 (27.8) | | |
| *ENPP1* | rs9373000 | 100 | AA | 49 (60.5) | 9 (47.4) | AA vs AG vs GG | 0.545 |
| | | | AG | 27 (33.3) | 8 (42.1) | | |
| | | | GG | 5 (6.2) | 2 (10.5) | | |
| | rs6569759 | 98 | GG | 19 (23.8) | 4 (22.2) | GG vs AG vs AA | 0.177 |
| | | | AG | 46 (57.5) | 7 (38.9) | | |
| | | | AA | 15 (18.7) | 7 (38.9) | | |
| | rs858339 | 93 | TT | 45 (59.2) | 6 (35.3) | TT vs AT vs AA | |
| | | | AT | 27 (35.5) | 10 (58.2) | | |
| | | | AA | 4 (5.3) | 1 (5.9) | | |
| | rs1409181 | 101 | CC | 13 (15.9) | 4 (21) | CC vs CG vs GG | 0.530 |
| | | | CG | 46 (58.1) | 12 (63.2) | | |
| | | | GG | 23 (28) | 3 (15.8) | | |
| *ESR1* | rs1643821 | 100 | GG | 33 (40.7) | 7 (36.9) | GG vs AG vs AA | 0.902 |

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| Gene | SNP | N | Genotype | <6 postoperative JPF score | ≥6 postoperative JPF score | Groups | p |
|---|---|---|---|---|---|---|---|
| | | | AG | 38 (46.9) | 10 (52.6) | | |
| | | | AA | 10 (12.4) | 2 510.5) | | |
| | rs3020318 | 99 | TT | 9 (11.2) | 5 (26.3) | TT vs CT vs CC | 0.175 |
| | | | CT | 37 (46.3) | 9 (47.4) | | |
| | | | CC | 34 (42.5) | 5 (26.3) | | |
| | rs3020377 | 99 | GG | 7 (8.6) | 3 (16.7) | GG vs AG vs AA | 0.139 |
| | | | AG | 31 (38.3) | 10 (55.5) | | |
| | | | AA | 43 (53.1) | 5 (27.8) | | |
| | rs2077647 | 101 | TT | 28 (34.2) | 4 (21.0) | TT vs CT vs CC | 0.511 |
| | | | CT | 41 (50) | 12 (63.2) | | |
| | | | CC | 13 (15.8) | 3 (15.8) | | |
| *PITX1* | rs1131611 | 100 | GG | 60 (74.1) | 16 (80.2) | GG vs GT vs TT | |
| | | | GT | 20 (24.7) | 2 (10.5) | | |
| | | | TT | 1 (1.2) | 1 (5.3) | | |
| *PITX2* | rs2595110 | 100 | AA | 43 (53.1) | 12 (63.2) | AA vs AG vs GG | 0.381 |
| | | | AG | 30 (37.0) | 4 (21.0) | | |
| | | | GG | 8 (9.9) | 3 (15.8) | | |

N : number of observation; my : mean ; DS : standard deviation; n (%) : number of observation (percentage); p : p-value

**Table 5**

Comparison of patients by Δ JPF score : >−2 Δ JPF group vs ≤ −2 Δ JPF *group (symptomatic worsening)*

| | >−2 Δ JPF N=78 | ≤ −2 Δ JPF N=23 | p |
|---|---|---|---|
| **Age** *my (DS)* | 26.0 (10.9) | 23.9 (12.6) | 0.208 |
| **Females** *n (%)* | 53 (68.0) | 14 (60.9) | 0.528 |
| ***Biometrical characteristics:*** | | | |
| **Sagittal** *n (%)* | | | |
| - Class II | 54 (69.2) | 16 (69.6) | 0.976 |
| - Class III | 24 (30.8) | 7 (30.4) | |
| **Vertical** *n (%)* | | | |
| - Normal Bite | 33 (42.3) | 7 (30.4) | 0.480 |
| - Open Bite | 30 (38.5) | 12 (57.2) | |
| - Deep Bite | 15 (19.7) | 4 (17.4) | |
| **Asymmetry** *n (%)* | | | |
| - No asymmetry | 29 (37.2) | 5 (21.7) | 0.168 |
| - Asymmetry | 49 (62.8) | 18 (78.3) | |
| **Me Angle** *my (DS)* | 85.4 (6.3) | 85.7 (6.2) | 0.833 |
| **Np Angle** *my (DS)* | 86.9 (4.1) | 87.1 (5.6) | 0.868 |
| **Adapted C1F1** *my (DS)* | 86.7 (2.8) | 87.7 (3.6) | 0.281 |
| **Facial height** (mm) *my (DS)* | 121.4 (9.6) | 120.8 (11.6) | 0.995 |
| **Bruxism** *n (%)* | 18 (23.1) | 3 (13.0) | 0.389 |
| ***Surgical characteristics:*** | | | |
| **Type of osteosynthesis :** | | | |
| Posterior rigid osteosynthesis | 56 (71.8) | 19 (82.6) | 0.297 |
| **Type of mandibular movement:** | | | |
| Mandibular advancement | 58 (74.4) | 16 (69.6) | 0.648 |

N : number of observation; my : mean ; DS : standard deviation; n (%) : number of observation (percentage); p : p-value

**Table 6**

Comparison of SNP genotypes by ΔJPF score : >−2 ΔJPF group vs ≤ −2 ΔJPF *(symptomatic worsening)*

| Gene | SNP | N | Genotype | >−2 ΔJPF | ≤ −2 ΔJPF | Groups | p |
|---|---|---|---|---|---|---|---|
| *ACTN3* | rs1671064 | 100 | AA n (%) | 16 (20.8) | 3 (13.0) | AA vs GA vs GG | 0.188 |
| | | | GA | 48 (62.3) | 12 (52.2) | | |
| | | | GG | 13 (16.9) | 8 (34.8) | | |
| | rs1815739 | 101 | CC | 18 (23.1) | 3 (13.1) | CC vs TC vs TT | 0.348 |
| | | | TC | 46 (59.0) | 13 (56.5) | | |
| | | | TT | 14 (18.0) | 7 (30.4) | | |
| | rs678397 | 95 | CC | 18 (24.7) | 3 (13.6) | CC vs CT vs TT | 0.101 |
| | | | CT | 41 (56.1) | 10 (45.5) | | |
| | | | TT | 14 (19.2) | 9 (40.9) | | |
| *ENPP1* | rs9373000 | 100 | AA | 44 (57.1) | 14 (60.9) | AA vs AG vs GG | 0.842 |
| | | | AG | 27 (35.1) | 8 (34.8) | | |
| | | | GG | 6 (7.8) | 1 (4.3) | | |
| | rs6569759 | 98 | GG | 18 (23.7) | 5 (22.7) | GG vs AG vs AA | 0.186 |
| | | | AG | 44 (57.9) | 9 (40.9) | | |
| | | | AA | 14 (18.4) | 8 (36.4) | | |
| | rs858339 | 93 | TT | 43 (56.6) | 8 (47.1) | TT vs AT vs AA | |
| | | | AT | 31 (40.8) | 6 (35.3) | | |
| | | | AA | 2 (2.6) | 3 (17.6) | | |
| | rs1409181 | 101 | CC | 12 (15.4) | 5 (21.7) | CC vs CG vs GG | 0.752 |
| | | | CG | 46 (59.0) | 12 (52.2) | | |
| | | | GG | 20 (25.6) | 6 (26.1) | | |
| *ESR1* | rs1643821 | 100 | GG | 34 (44.2) | 6 (26.1) | GG vs AG vs AA | **0.041** |

| Gene | SNP | N | Genotype | >−2 ΔJPF | ≤ −2 ΔJPF | Groups | p |
|---|---|---|---|---|---|---|---|
| | | | AG | 37 (48.0) | 11 (47.8) | | |
| | | | AA | 6 (7.8) | 6 (26.1) | | |
| | rs3020318 | 99 | | | | | |
| | | | TT | 10 (13.1) | 4 (17.4) | TT vs CT vs CC | 0.869 |
| | | | CT | 36 (47.4) | 10 (43.5) | | |
| | | | CC | 30 (39.5) | 9 (39.1) | | |
| | rs3020377 | 99 | | | | | |
| | | | GG | 8 (10.4) | 2 (9.2) | GG vs AG vs AA | 0.908 |
| | | | AG | 31 (40.3) | 10 (45.4) | | |
| | | | AA | 38 (49.3) | 10 (45.4) | | |
| | rs2077647 | 101 | | | | | |
| | | | TT | 24 (30.1) | 8 (34.8) | TT vs CT vs CC | 0.215 |
| | | | CT | 44 (56.4) | 9 (39.1) | | |
| | | | CC | 10 (12.8) | 6 (26.1) | | |
| *PITX1* | rs1131611 | 100 | | | | | |
| | | | GG | 58 (75.3) | 18 (78.3) | GG vs GT vs TT | |
| | | | GT | 18 (23.4) | 4 (17.4) | | |
| | | | TT | 1 (1.3) | 1 (4.3) | | |
| *PITX2* | rs2595110 | 100 | | | | | |
| | | | AA | 42 (54.5) | 13 (56.5) | AA vs AG vs GG | 0.922 |
| | | | AG | 26 (33.8) | 8 (34.8) | | |
| | | | GG | 9 (11.7) | 2 (8.7) | | |

N : number of observation; my : mean ; DS : standard deviation; n (%) : number of observation (percentage); p : p-value

**Table 7**

Comparison of rs1643821 genotypes (*ESR1* gene) by ΔJPF score: >−2 ΔJPF score group vs ≤ −2 ΔJPF score group (symptomatic worsening)

| SNP | N | Genotype | ≤ −2 ΔJPF | >−2 ΔJPF | Groups | p | OR | 95% IC | |
|---|---|---|---|---|---|---|---|---|---|
| rs1643821 | 100 | GG n (%) | 6 (26.1) | 34 (44.2) | GG vs AG vs AA | **0.041** | | | |
| | | AG | 11 (47.8) | 37 (48.0) | | | | | |
| | | AA | 6 (26.1) | 6 (7.8) | | | | | |
| | | GG | 6 (26.1) | 34 (44.2) | GG vs (AG + AA) | 0.121 | 0.446 | 0.159 | 1.255 |
| | | AG + AA | 17 (73.9) | 43 (55.8) | | | | | |
| | | AG | 11 (47.8) | 37 (48.1) | AG vs (GG + AA) | 0.985 | 0.991 | 0.390 | 2.517 |
| | | GG + AA | 12 (52.2) | 42 (51.9) | | | | | |
| | | AA | 6 (26.1) | 6 (7.8) | AA vs (GG +AG) | **0.028** | **4.176** | **1.198** | **14.566** |
| | | GG + AG | 17 (73.9) | 71 (92.2) | | | | | |

N : number of observation; n (%) : number of observation (percentage) ; p : p-value ; OR : odds ratio ; 95% IC : confiderce interval of 95%

**Table 8**

Comparison of patients by preoperative JPF score : <6 JPF score group vs ≥6 JPF score group (preoperative TMD)

| | <6 JPF score N=74 | ≥6 JPF score N=27 | p |
|---|---|---|---|
| **Age** *my (DS)* | 24.4 (11.5) | 28.7 (10.3) | 0.018 |
| **Females** *n (%)* | 44 (59.5) | 23 (85.2) | 0.015 |
| ***Biometrical characteristics:*** | | | |
| **Sagittal** *n (%)* | | | |
| - Class II | 49 (66.2) | 21 (77.8) | 0.265 |
| - Class III | 25 (33.8) | 6 (22.2) | |
| **Vertical** *n (%)* | | | |
| - Normal Bite | 29 (39.2) | 11 (40.7) | 0.990 |
| - Open Bite | 31 (41.9) | 11 (40.7) | |
| - Deep Bite | 14 (18.9) | 5 (18.6) | |
| **Asymmetry** *n (%)* | | | |
| - No asymmetry | 24 (32.4) | 10 (34.0) | 0.665 |
| - Asymmetry | 50 (67.6) | 17 (63.0) | |
| **Me Angle** *my (DS)* | 85.7 (6.2) | 84.7 (6.5) | 0.225 |
| **Np Angle** *my (DS)* | 87.1 (4.6) | 86.6 (4.2) | 0.611 |
| **Adapted C1F1** *my (DS)* | 87.2 (3.2) | 86.1 (2.4) | 0.142 |
| **Facial height** (mm) *my (DS)* | 122.0 (10.5) | 119.5 (8.4) | 0.271 |
| **Bruxism** *n (%)* | 11 (14.9) | 10 (37.0) | 0.015 |

N : number of observation; my : mean ; DS : standard deviation; n (%) : number of observation (percentage); p : p-value

**Table 9**

Comparison of SNP genotypes by preoperative JPF score: <6 preoperative JPF score group vs ≥6 JPF score group (preoperative TMD)

| Gene | SNP | N | Genotype | <6 preoperative JPF score | ≥6 preoperative JPF score | Groups | p |
|---|---|---|---|---|---|---|---|
| *ACTN3* | rs1671064 | 100 | AA n (%) | 13 (17.8) | 6 (22.2) | AA vs GA vs GG | 0.333 |
| | | | GA | 42 (57.5) | 18 (66.7) | | |
| | | | GG | 18 (54.7) | 3 (11.1) | | |
| | rs1815739 | 101 | CC | 15 (20.3) | 6 (22.2) | CC vs TC vs TT | 0.345 |
| | | | TC | 41 (55.4) | 18 (66.7) | | |
| | | | TT | 18 (24.3) | 3 (11.1) | | |
| | rs678397 | 95 | CC | 15 (21.4) | 6 (24) | CC vs CT vs TT | 0.244 |
| | | | CT | 35 (50) | 16 (64) | | |
| | | | TT | 20 (28.6) | 3 (12) | | |
| *ENPP1* | rs9373000 | 100 | AA | 44 (60.3) | 14 (51.9) | AA vs AG vs GG | 0.555 |
| | | | AG | 25 (34.2) | 10 (37.0) | | |
| | | | GG | 4 (5.5) | 3 (11.1) | | |
| | rs6569759 | 98 | GG | 16 (22.5) | 7 (25.9) | GG vs AG vs AA | 0.937 |
| | | | AG | 39 (55.0) | 14 (51.9) | | |
| | | | AA | 16 (22.5) | 6 (22.2) | | |
| | rs858339 | 93 | TT | 42 (63.6) | 9 (33.3) | TT vs AT vs AA | **0.014** |
| | | | AT | 20 (30.3) | 17 (63.0) | | |
| | | | AA | 4 (6.1) | 1 (3.7) | | |
| | rs1409181 | 101 | CC | 11 (14.9) | 6 (22.2) | CC vs CG vs GG | 0.658 |
| | | | CG | 43 (58.1) | 15 (55.6) | | |
| | | | GG | 20 (27.0) | 6 (22.2) | | |
| *ESR1* | rs1643821 | 100 | GG | 28 (38.4) | 12 (44.4) | GG vs AG vs AA | 0.859 |

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| Gene | SNP | N | Genotype | <6 preoperative JPF score | ≥6 preoperative JPF score | Groups | p |
|---|---|---|---|---|---|---|---|
| | | | AG | 36 (49.3) | 12 (44.4) | | |
| | | | AA | 9 (12.3) | 3 (11.2) | | |
| | rs3020318 | 99 | | | | | |
| | | | TT | 10 (13.9) | 4 (14.8) | TT vs CT vs CC | 0.745 |
| | | | CT | 32 (44.4) | 14 (51.9) | | |
| | | | CC | 30 (41.7) | 9 (33.3) | | |
| | rs3020377 | 99 | | | | | |
| | | | GG | 6 (8.2) | 4 (15.4) | GG vs AG vs AA | 0.500 |
| | | | AG | 32 (43.8) | 9 (34.6) | | |
| | | | AA | 35 (48.0) | 13 (50) | | |
| | rs2077647 | 101 | | | | | |
| | | | TT | 25 (33.8) | 7 (25.9) | TT vs CT vs CC | 0.734 |
| | | | CT | 38 (51.3) | 15 (55.6) | | |
| | | | CC | 11 (14.9) | 5 (18.5) | | |
| *PITX1* | rs1131611 | 100 | | | | | |
| | | | GG | 58 (78.4) | 18 (69.2) | GG vs GT vs TT | |
| | | | GT | 15 (20.3) | 7 (26.9) | | |
| | | | TT | 1 (1.3) | 1 (3.9) | | |
| *PITX2* | rs2595110 | 100 | | | | | |
| | | | AA | 38 (52.1) | 17 (63.0) | AA vs AG vs GG | 0.586 |
| | | | AG | 26 (35.6) | 8 (29.6) | | |
| | | | GG | 9 (12.3) | 2 (7.4) | | |

N : number of observation; my : mean ; DS : standard deviation; n (%) : number of observation (percentage); p : p-value

**Table 10**

Comparison of rs858339 genotypes (*ENPP1* gene) by preoperative JPF score: <6 preoperative JPF score group vs ≥6 preoperative JPF score group (TMD)

| SNP | N | Genotype | <6 preoperative JPF score | ≥6 preoperative JPF score | Groups | p | OR | 95% IC | |
|---|---|---|---|---|---|---|---|---|---|
| rs858339 | 93 | TT n (%) | 42 (63.6) | 9 (33.3) | TT vs AT vs AA | **0.014** | | | |
| | | AT | 20 (30.3) | 17 (63.0) | | | | | |
| | | AA | 4 (6.1) | 1 (3.7) | | | | | |
| | | TT | 42 (63.6) | 9 (33.3) | TT vs (AT + AA) | **0.008** | 0.286 | 0.111 | 0.735 |
| | | AT + AA | 24 (36.4) | 18 (66.7) | | | | | |
| | | AT | 20 (30.3) | 17 (63.0) | AT vs (TT + AA) | **0.003** | 3.910 | 1.526 | 10.021 |
| | | TT + AA | 46 (69.7) | 10 (37.0) | | | | | |
| | | AA | 4 (6.1) | 1 (3.7) | AA vs (TT + AT) | | | | |
| | | TT + AT | 62 (93.9) | 26 (96.3) | | | | | |

N : number of observation; n (%) : number of observation (percentage) ; p : p-value ; OR : odds ratio ; 95% IC : confidence interval of 95%

## II. Génotypes d'*ENPP1 et* variation de la géométrie condylienne

INTRODUCTION : Le remodelage osseux permet une adaptation du condyle mandibulaire, il participe à la stabilité morphologique, fonctionnelle et occlusale. Le gène *ENPP1* contribue à la morpho-biométrie osseuse et à la densité minérale osseuse, l'objectif de cette étude était d'étudier l'association entre les polymorphismes nucléotidiques du gène *ENPP1* et le remodelage condylien.

METHODE : 156 patients ayant bénéficié d'un traitement orthodontico-chirurgical pour corriger une dysmorphose dentofaciale ont été inclus dans cette étude. Des échantillons de salive étaient recueillis pour chaque sujet afin d'extraire et de génotyper leur ADN. Quatre polymorphismes du gène *ENPP1* ont été sélectionnés et testés pour rechercher une association entre des variants alléliques spécifiques et le remodelage condylien. Les critères de remodelage condylien choisis étaient le différentiel de hauteur condylienne et de surface de l'unité condylienne, mesurés sur le panoramique dentaire de chaque patient. Un seuil de significativité à 15% de différence était retenu.

RESULTATS : La répartition entre les groupes étaient comparables pour l'âge, le sexe et les caractéristiques biométriques. Le polymorphisme nucléotidique rs9373000 du gène *ENPP1* était statistiquement associé à un différentiel de hauteur >15% entre les deux condyles d'un même patient (p=0.012). Le génotype GG était retrouvé comme un facteur protecteur de la diminution de hauteur de l'unité condylienne (p=0.003).

CONCLUSION : Cette étude a mis en évidence une relation entre le variant rs9373000 et la morphologie du condyle mandibulaire chez les patients présentant une dysmorphose dentofaciale.

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# Condylar geometry variation is associated with *ENPP1* variant in a population of patients with dento-facial deformities

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## Summary

**Purpose**—Bone remodeling is essential in maintaining bone health. Considering that *ENPP1* contributes to bone geometry and bone mineralization, the aim of our study was to analyze the association between single-nucleotide polymorphisms (SNPs) of *ENPP1* and condylar remodeling.

**Materials and Methods**—A total of 156 patients undergoing orthodontic and maxillofacial surgery treatment for correction of malocclusion were included in this prospective study. Saliva samples from all subjects were used for DNA extraction and genotyping. Four *ENPP1* SNP*s* were selected and tested to determine whether specific allelic variants are correlated with condylar

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University of Lille CPP (Committee for Personal Protection): CPP12/44

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remodeling. The criteria of condylar remodeling chosen were the ratio between each side of condylar height or surface differences on a dental panoramic of each patient. A diagnostic threshold was set at 15% difference between both sides.

**Results**—The *ENPP1* SNP rs9373000 showed a statistically significant association with condylar height ratio > 15% (p=0.012). The GG genotype was found to be a protective factor against condylar height decrease (p=0.003).

**Conclusion**—This study identifies the genetic variant rs9373000 as a potentially causal variant for mandibular condyle geometry variation for patients presenting with dento-facial deformities.

**Keywords**

ectonucleotide pyrophosphatase phosphodiesterase 1; mandibular condyle; temporomandibular joint; malocclusion

## Introduction

The temporomandibular joint is an adaptive structure that is capable of anatomic modification to ensure morphological, functional, and occlusal stability. This phenomenon is often termed "condylar remodeling," which occurs in response to genetic, demographic, anthropometric, hormonal, or dysfunctional etiologic influences (Arnett *et al.*, 1996a, 1996b).

Few studies have focused on genes involved in remodeling of the condylar unit, since there is a lack of reproducible diagnostic criteria. The literature identifies several remodeling criteria, including a decrease in condylar head volume, a decrease of condylar unit and mandibular rami height, and changes in mandibular shape and class II malocclusion (Hwang *et al.*, 2004; Huang *et al.*, 1997). These criteria are difficult to identify on standard radiographs, making a phenotypic subclassification of condylar remodeling for genotype associations difficult.

Mineralization of the extracellular matrix is essential for bone growth, bone stability, and bone turnover. The balance between levels of inorganic phosphate and pyrophosphate are essential to the quality of mineralization. During the mineralization process, hydroxyapatite is formed by the crystallization of calcium and inorganic phosphate. *ENPP1* (ectonucleotide pyrophosphatase / phosphodiesterase 1) is a gene encoding a transmembrane ectoenzyme, controlling this process by hydrolysis of inorganic pyrophosphate and therefore inhibiting hydroxyapatite formation. The Framingham cohort study (Cheung *et al.*, 2010) identified associations between single-nucleotide polymorphisms (SNPs) in the *ENPP1* gene with bone morpho-biometrics, such as changes in hip geometry. Subsequent studies identified similar associations in *ENPP1* SNPs with variations in facial bone morpho-biometrics and class III malocclusion (Deeley *et al.*, 2015). Rs858339 (*ENPP1)* has recently been associated with temporomandibular dysfunctions in a population with dento-facial deformities (Nicot *et al.*, 2016). Therefore *ENPP1* is a gene of interest in mandibular condylar bone morphometry. In this study, we investigated the association between SNPs of *ENPP1* and pre-surgical morphometric landmarks associated with condylar remodeling, using measurable diagnostic criteria. Condylar height and surface ratios of the condylar unit were used to evaluate

condylar remodeling, given their reproducibility as biometric measurements on pre-surgical panoramic radiographs.

## Materials and Methods

Consecutive subjects undergoing orthodontic and maxillofacial surgery treatment for correction of malocclusion were recruited between February 2013 and June 2014 from the Department of Oral and Maxillofacial Surgery at the University of Lille in France. Patients were adults or non-growing adolescents who had no past history of mandibular trauma, systemic conditions or congenital malformation syndromes that might influence condylar resorption. No patients included showed significant condylar remodeling during presurgical orthodontic treatment. Subjects signed an informed consent form, and the research protocol was validated by the French independent ethical committee, the Temple University and the University of Pittsburgh institutional review boards.

Age, sex, facial biometrics and TMJ symptoms were listed during the preoperative examination. Cephalometric data and classification were obtained applying the Delaire (Delaire *et al.*, 1981) analysis method on presurgical radiographs. Given the lack of repeatable measurable criteria for condylar remodeling in the literature, we have chosen as primary endpoints t o measure the ratio between each side of condylar height or surface on presurgical dental panoramic radiographs, carried out according to the same protocol. Two lines were constructed to evaluate condylar height, one drawn tangential to the posterior edge of the mandible passing through the most posterior points of the condyle and mandibular ramus, and the perpendicular line passing through the lower end of the mandibular notch. Condylar height was measured perpendicular to the latter between the mandibular notch and the highest point of the condylar unit (Figure 1A). The surface of the condylar unit was measured using computer software, contouring the lowest point of the mandibular notch to the *lingula mandibulae*, then perpendicular to the rear edge of the mandibular ramus (Figure 1B). Bone remodeling has been determined in this study by a differential measurement of height or condylar surface defined by a percentage in relation to the larger side between right and left sides of presurgical dental panoramic. Diagnosis of remodeling was set at a 15% differential for comparison to data found in the literature.

Saliva samples from all subjects were stored in Oragene kits (DNA Genotek, Ottawa, ON, Canada) and used for DNA extraction and posterior genotyping. Four SNPs of *ENPP1* (rs9373000, rs6569759, rs858339 and rs1409181) were selected for genotyping and tested to determine whether specific allelic variants correlated with condylar biometries using TaqMan chemistry (Life Technologies, Carlsbad, CA, USA) and end-point analysis in an automatic sequence-detection instrument (ABI Prism 7900HT; Applied Biosystems, Foster City, CA, USA), as described previously (Zucchero *et al.*, 2004). SNPs chosen to study ENPP1 had a minor allele frequency (MAF) ≥ 5% and were located in or flanking the gene, with HWE $p \leq 0.001$ and $r^2 \geq 0.70$ based on HapMap Phase II, in a northwest European population (Cheung *et al.*, 2010).

The characteristics of the population were presented with the usual rules of descriptive statistics. After ensuring compliance with Hardy-Weinberg equilibrium, the association

between SNPs and condylar height or surface ratios was sought by the Chi-squared test. We divided the analysis population into two groups depending on the surface or height ratios of condylar unit. Considering a threshold of 15% for both primary endpoints, we obtained a qualitative variable in two groups. Patients with condylar surface ratio < 15% (Diff Surf < 15% group) represented the subpopulation without condylar remodeling while patients with condylar surface ratio ≥ 15% (Diff Surf ≥ 15%) were classified in the subpopulation with a potential condylar remodeling. In the same way, patients with condylar height ratio < 15% (Diff Ht < 15% group) represented the subpopulation without condylar remodeling, while patients with condylar height ratio ≥ 15% (Diff Ht ≥ 15%) were classified in the subpopulation with potential condylar remodeling. For each SNP, analysis was performed by considering the three genotypes separately. In case of significance, analysis was completed to compare each genotype to the two others, and odds ratios (OR) with 95% confidence intervals (95% CI) were calculated. The significance level was set at $p = 0.05$. It was recorded in the tables when the Chi-squared test conditions were not met.

## Results

A total of 156 were included in the study between February 2013 and June 2014. This sample included a larger proportion of women (68.6%), young people (25.8 ±11.0 years) and Class II dentofacial deformity patients (68%). Age, sex and biometric characteristics of the population are listed in Table 1. The comparative analysis based on condylar height or surface ratios iudicated that there were no significant differences between the two groups for age, sex or biometric characteristics of patients, suggesting that the two populations were similar. We found 112 patients with condylar surface ratio < 15% and 22 patients with condylar surface ratio ≥ 15%. For 22 of 156 patients, the condylar surface ratio was not measurable. A total of 113 patients presented with a condylar height ratio < 15%, and 20 patients had a condylar height ratio ≥ 15%. For 23 patients, the condylar height ratio could not be calculated. No SNP was found to be statistically associated with condylar surface ratio (Table 2). On the other hand, single-nucleotide polymorphism rs9373000 of *ENPP1* presented a statistically significant association with condylar height ratio (p = 0.012) (Table 3). In particular, the GG genotype was found to be a protective factor against condylar height decrease (OR = 0.14, 95% C.I. 0.082-0.240, p = 0.003) (Table 4).

## Discussion

This study highlights the significant association between single nucleotide polymorphism rs9373000 of *ENPP1* and condylar height ratio ($p = 0.012$). The results are consistent with the analysis of the literature pointing out the role of *ENPP1* in bone geometry and bone mineralization.

*ENPP1* encodes a transmembrane ecto-enzyme hydrolyzing extracellular molecules having pyrophosphate or phosphodiester bonds. In chondrocytes and osteoblasts, it regulates bone mineralization (Harmey *et al.*, 2004). Specifically, it hydrolyzes inorganic pyrophosphate (PPi), one of the main inhibitors of calcification, which inhibits hydroxyapatite crystal growth (Terkeltaub, 2001). The role of *ENPP1* in inhibiting calcification was initially demonstrated in a murine model of ttw / ttw phenotype (tiptoe walking), which carries a

nonsense mutation in the *Enpp1* (Hajjawi *et al.*, 2014). The mice *Enpp1*−/− developed a phenotype of hypermineralization, with calcification of tendons and ligaments. These *Enpp1*−/− mice are a particular model for the study of the human disease, which is responsible for the ossification of posterior longitudinal ligament. Phenotypic characteristics of *Enpp1*−/− mice include marked alterations in mineralization of long bones and skull, and the pathological calcification of perispinal soft tissues and medial arterial layer. In human pathology, *ENPP1* gene mutation produces generalized arterial calcification and pseudoxanthoma elasticum in children (Nitschke *et al.*, 2012).

Regarding gene polymorphisms, the SNP rs1974201 has been identified as a potentially causal variant of bone geometry in the Framingham cohort study (Cheung *et al.*, 2010). *ENPP1* has also become a potential gene of interest in the field of craniofacial bone morphometry. In particular, an association has been observed between upper facial height and polymorphisms located near the promoter region and upstream from *ENPP1* (Ermakov *et al.*, 2010a). Deeley *et al.* (Deeley *et al.*, 2015) have also shown an association between rs9373000 and rs6569759 and mandibular prognathism in subjects with dento-facial deformities, suggesting a role of *ENPP1* in facial bone geometry. On the other hand, rs858339 (*ENPP1)* has recently been associated with temporomandibular dysfunctions in a population with dento-facial deformities, which highlights its morpho-functional role (Nicot *et al.*, 2016). Apart from their role in facial bone geometry, several polymorphisms of *ENPP1* were associated with changes in long bone geometry (Cheung *et al.*, 2010; Ermakov *et al.*, 2010b). It has particularly been shown that the association between bone size phenotypes and the distal segment of *ENPP1* imply functional significance of its 3′ untranslated region to bone growth (Ermakov *et al.*, 2010b). *ENPP1* polymorphisms also have been associated with radiographic hand osteoarthritis (Suk *et al.*, 2005).

This study identifies the genetic variant rs9373000 in *ENPP1* as a potentially causal variant for mandibular condyle geometry variation in a cohort of patients presenting with dento-facial deformities. Despite not using the Bonferroni correction, the observed association in this study is unlikely to be a spurious finding, since 0.05 divided by four SNP markers would reduce the p-value to 0.0125. Resorption of the mandibular condyle has multifactorial origins (Ferri *et al.*, 2016). The main demographic and biometric factors influencing condylar resorptions are young age, female sex, Class II dentofacial deformity and open-bite malocclusion. These factors are strongly represented in our population. Indeed, the sample included a larger proportion of women (68.6%), young people (25.8 ± 11.0 years) and patients with Class II dentofacial deformity (68%) or open-bite malocclusion (45.5%). Distribution of these factors is, however, similar in both groups, depending on condylar surface ratio or condylar height ratio (patients with condylar surface ratio < 15% and those with condylar surface ratio > 15%, or patients with condylar height ratio < 15% and those with condylar height ratio > 15%). In other words, both study groups were comparable for age, sex and biometric characteristics, limiting the confounders even if we have not performed a multivariate analysis. This is particularly important, given the fact that young age, female sex and some biometric traits such as Class 2 or open-bite malocclusions are factors associated with condylar resorption and condylar remodeling (Wolford and Cardenas, 1999; Papadaki *et al.*, 2007).

The primary endpoints chosen (condylar surface ratio and condylar height ratio) have the benefit of being measurable and therefore reproducible, unlike conventional criteria for condylar resorption found in the literature, which sometimes leaves room for interpretation. Using an orthopantomogram instead of a cone beam for implementing the measures can be considered a limitation of our study. Comparison of the two condyles of a patient on the same radiography limits the measurement bias but poses the problem of the exclusion of bilateral and symmetrical condylar remodeling. Hlawitschka and Eckelt also used panoramic radiographs to compare the two condyles of the same patient with condylar fractures and to measure the loss of condylar height compared with the healthy side (Hlawitschka and Eckelt, 2002). We have chosen a differential of 15 ° in order to limit false-positive results, while Habets *et al.* diagnosed a condylar remodeling when there was a height differential of > 6° (Habets *et al.*, 1987; Habets *et al.*, 1988). Considering the lack of power of our study, these results will have to be verified with a larger sample.

Our results suggest that rs9373000 variant is associated with condylar height ratio (p = 0.012). In particular, the GG genotype was identified as a protective factor against condylar height reduction (OR = 0.14, 95% CI 0.082-0.240, p = 0.003). How this SNP alters bone metabolism to affect bone formation remains unknown. rs9373000 is an *ENPP1* variant mapped on the 3′UTR (Mignone *et al.*, 2002). Single nucleotide polymorphisms falling specifically in the 3′UTR of genes may interfere with mRNA stability and translation through effects on polyadenylation and regulatory protein-mRNA and miRNA-mRNA interactions (Arnold *et al.*, 2012). For example, previous studies in humans have identified variations in the 3′UTR of genes that appear to affect cancer risk by disrupting normal miRNA binding (Nicoloso *et al.*, 2012). Similarly, with some variations in the 3′UTR of genes affecting cancer risk by disrupting normal miRNA binding, the rs9373000 variant could be another example of polymorphism affecting the 3′UTR of genes by generating a specific modulation of bone geometry. Our results further reinforce those of Ermakov *et al.* regarding the role of the 3′UTR of *ENPP1* in bone geometry (Ermakov *et al.*, 2010b).

These findings are particularly interesting, given the morpho-functional role of the temporomandibular joint. It is clear that a change, even moderate, of mandibular condyle geometry can influence the proper functionality of this joint. In particular, the measurement criteria used are good reflections of the condylar remodeling, emphasizing the morpho-functional role of the variant rs9373000 in the craniofacial field. Bone mineral density and mandibular advancement have been recently identified as contributing factors for postoperative relapse after orthognathic surgery in patients with preoperative idiopathic condylar resorption (Yang and Hwang, 2015). Under these conditions, *ENPP1* could represent a potential gene of interest regarding relapse in orthognathic surgery patients with idiopathic condylar resorption (Hoppenreijs *et al.*, 2013). This study has been performed in a population of patients with dento-facial deformities requiring orthognathic surgery. Therefore, this study design allows us to investigate this potential gene of interest in orthognathic surgery where it could represent a potential gene of interest regarding relapse, in particular in the context of preoperative idiopathic condylar resorption.

## Conclusion

In conclusion, we found a significant association between single nucleotide polymorphism rs9373000 of *ENPP1* and condylar height ratio, highlighting its morphofunctional role. The GG genotype was found to be a potential protective factor against condylar height decrease (OR = 0.14, 95% CI 0.082-0.240, p = 0.003). This preliminary work leads us to initiate studies of condylar remodeling or resorption and *ENPP1* variants. The next step in this cohort study will be to study the link between *ENPP1* and postoperative condylar resorption after bilateral sagittal split osteotomy.

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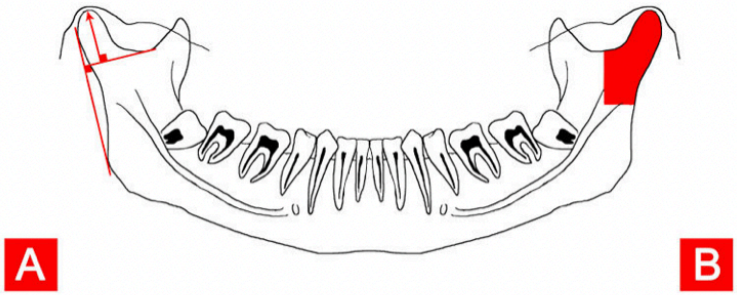


**Figure 1.**
Panoramic landmarks. (A) Condylar height measurement. (B) Condylar surface measurement.

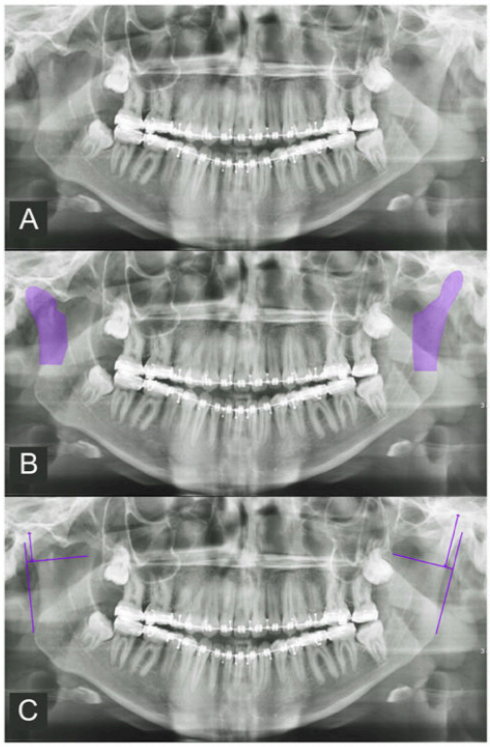


**Figure 2.**
Implementation of the measures in a patient with a right condylar remodeling. Surface differential measured at 22.22%. Height differential measured at 53.11%.

**Table 1**

**Description of analysis population**

| Characteristics of analysis population | N=156 |
|---|---|
| **Age** *my* (DS) | 25,8 (11,0) |
| **Females** *n* (%) | 107 (68,6) |
| ***Biometrical characteristics:*** | |
| **Sagittal** *n* (%) | |
| - Class I | 1 (0,6) |
| - Class II | 106 (68,0) |
| - Class III | 49 (31,4) |
| **Vertical** *n* (%) | |
| - Normal bite | 51 (32,7) |
| - Open bite | 71 (45,5) |
| - Deep bite | 34 (21,8) |
| **Mandibular asymmetry** *n* (%) | |
| - No asymmetry | 99 (63,5) |
| - Mandibular asymmetry > 2mm | 57 (36,5) |

N : number of observation; my : mean; DS : standard deviation; n (%) : number of observation (percentage)

**Table 2**

**Comparison of SNP genotypes by condylar surface ratio (*DIff Surf*): *Diff Surf < 15% group vs Diff Surf ≥ 15%***

| Gene | SNP | N | Genotype | Diff Surf < 15% | Diff Surf ≥ 15% | Groups | p |
|---|---|---|---|---|---|---|---|
| | | | | n (%) | n (%) | | |
| *ENPP1* | rs9373000 | 134 | AA | 59 (52,7) | 14 (63,6) | AA vs AG vs GG | 0,592 * |
| | | | AG | 43 (38,4) | 7 (31,8) | | |
| | | | GG | 10 (8,9) | 1 (4,6) | | |
| | rs6569759 | 131 | GG | 26 (23,6) | 8 (38,1) | GG vs AG vs AA | 0,184 * |
| | | | AG | 56 (50,9) | 11 (52,4) | | |
| | | | AA | 28 (25,5) | 2 (9,5) | | |
| | rs858339 | 113 | TT | 50 (52,1) | 11 (64,7) | TT vs AT vs AA | 0,472 * |
| | | | AT | 41 (42,7) | 6 (35,3) | | |
| | | | AA | 5 (5,2) | 0 (0) | | |
| rs1409181 | 134 | CC | 20 (17,9) | 3 (13,6) | CC vs CG vs GG | 0,763 * | |
| | | CG | 64 (57,1) | 12 (54,6) | | | |
| | | GG | 28 (25) | 7 (31,8) | | | |

N : number of observation; n (%) : number of observation (percentage);

* Chi-2 test conditions were not met

*J Craniomaxillofac Surg*. Author manuscript; available in PMC 2018 June 01.

**Table 3**

**Comparison of SNP genotypes by condylar height ratio (*DIff Ht*): *Diff Ht < 15% group vs Diff Ht ≥ 15%***

| Gene | SNP | N | Genotype | Diff Ht < 15% | Diff Ht ≥ 15% | Groups | p |
|---|---|---|---|---|---|---|---|
| *ENPP1* | rs9373000 | 133 | AA | 64 (56,6) | 8 (40) | AA vs AG vs GG | **0,012** |
| | | | AG | 43 (38,1) | 7 (35) | | |
| | | | GG | 6 (5,3) | 5 (25) | | |
| | rs6569759 | 130 | GG | 31 (27,9) | 2 (10,5) | GG vs AG vs AA | 0,270 * |
| | | | AG | 55 (49,6) | 12 (63,2) | | |
| | | | AA | 25 (22,5) | 5 (26,3) | | |
| | rs858339 | 113 | TT | 52 (54,2) | 9 (52,9) | TT vs AT vs AA | 0,597 * |
| | | | AT | 39 (40,6) | 8 (47,1) | | |
| | | | AA | 5 (5,2) | 0 (0,0) | | |
| rs1409181 | 133 | CC | 20 (17,7) | 3 (15) | CC vs CG vs GG | 0,873 * | |
| | | CG | 65 (57,5) | 11 (55) | | | |
| | | GG | 28 (24,8) | 6 (30) | | | |

N : number of observation; n (%) : number of observation (percentage);

* Chi-2 test conditions were not met

*J Craniomaxillofac Surg*. Author manuscript; available in PMC 2018 June 01.

**Table 4**

**Comparison of rs9373000 genotypes of *ENPP1* gene by condylar height ratio (*Diff Ht*): *Diff Ht < 15% group vs Diff Ht ≥ 15%***

| SNP | N | Génotype | Diff Ht < 15% | Diff Ht ≥ 15% | Groups | p | OR | IC 95% |
|---|---|---|---|---|---|---|---|---|
| rs9373000 | 133 | AA | 64 (56,6) | 8 (40) | AA vs AG vs GG | **0,012** | | |
| | | AG | 43 (38,1) | 7 (35) | | | | |
| | | GG | 6 (5,3) | 5 (25) | | | | |
| | | | | | AA vs (AG + GG) | 0,169 | 0.125 | 0.060 0.261 |
| | | | | | AG vs (AA + GG) | 0,795 | 0.186 | 0.103 0.336 |
| | | | | | GG vs (AA +AG) | **0,003** | **0,140** | **0.082 0.240** |

SNP : single-nucleotid polymorphism; N : number of observation; n (%) : number of observation (percentage); OR : odds ratio; 95% IC : confidence interval of 95%

## III. Relation entre les génotypes d'*ENPP1* et d'*ESR1*, les sous-classifications d'asymétrie craniofaciale et les dysfonctions temporomandibulaires avant et après traitement chirurgical des dysmorphoses dentofaciales

INTRODUCTION : L'objectif de cette étude était d'évaluer si les polymorphismes des gènes *ACTN3*, *ENPP1*, *ESR1*, *PITX1* et *PITX2* contribuent aux asymétries faciales et aux dysfonctions temporomandibulaires avant et après une prise en charge orthodontico-chirurgicale.

METHODE : Cent soixante-quatorze patients présentant une dysmorphose dentofaciale ont été diagnostiqués comme symétriques ou subdivisés en 4 groupes asymétriques selon les mesures céphalométriques postéro-antérieures. Un diagnostic clinique et par le biais du questionnaire sur la douleur et la fonction des mâchoires (JPF) ont permis d'évaluer la présence et la gravité d'une dysfonction temporomandibulaire.

RESULTATS : Cinquante-deux pour cent des patients étaient symétriques, et 48 % étaient asymétriques. La classification de l'asymétrie a montré des différences céphalométriques significatives entre les groupes symétriques et asymétriques, et entre les 4 sous-types d'asymétrie : groupe 1, asymétrie du corps mandibulaire ; groupe 2, asymétrie du ramus ; groupe 3, asymétrie atypique ; et groupe 4, asymétrie en forme de C. Le SNP rs6569759 d'*ENPP1* était associé au groupe 1 ($p = 0,004$), et le rs858339 était associé au groupe 3 ($p = 0,002$). Le SNP rs164321 d'*ESR1* était associé au groupe 4 ($p = 0,019$). Ces résultats ont été confirmés par l'analyse en composantes principales qui a montré que 3 composantes principales expliquent près de 80% des variations dans les groupes étudiés. Les composantes principales 1 et 2 étaient associées au SNP rs3020318 d'*ESR1* ($p < 0,05$). Les diagnostics de déplacement discal réductible, de myalgie des muscles masticateurs et d'arthralgie étaient très répandus dans les groupes d'asymétrie, et tous présentaient des associations statistiques fortes avec le polymorphisme rs858339 du gène *ENPP1*. Les scores moyens de JPF des sujets asymétriques avant la chirurgie (JPF, 7) étaient significativement plus élevés que ceux des sujets symétriques (JPF, 2). Les patients du groupe 3 avaient les scores JPF préopératoires les plus élevés, et les groupes 2 et 3 étaient les plus susceptibles d'être guéris d'une dysfonction temporomandibulaire 1 an après le traitement.

CONCLUSION : La céphalométrie postéro-antérieure permet de classer l'asymétrie en groupes distincts et d'identifier la probabilité d’une dysfonction temporomandibulaire et les associations de génotypes. La prise en charge orthodontico-chirurgicale de l'asymétrie faciale est efficace dans le traitement des dysfonctions temporomandibulaires chez la plupart des patients.

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# ENPP1 and ESR1 Genotypes Associate with Subclassifications of Craniofacial Asymmetry and Severity of TMD

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Professor Sciote – Overall project supervisor and principle writer.
All authors participated in writing of the manuscript. All authors have approved the final article.



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## Abstract

**Introduction—**We investigated if *ACTN3*, *ENPP1*, *ESR1*, *PITX1* and *PITX2* genes which contribute to sagittal and vertical malocclusion also contribute to facial asymmetries and TMD before and after orthodontic and orthognathic surgery treatment.

**Methods—**One hundred seventy four dentofacial deformity patients were diagnosed as symmetric or subdivided into four asymmetric groups according to PA cephalometric measurements. TMD exam diagnosis and Jaw Pain and Function-(JPF) questionnaires assessed presence and severity of TMD.

**Results—**Fifty two % of patients were symmetric and forty eight % asymmetric. The asymmetry classification demonstrated significant cephalometric differences between symmetric and asymmetric groups, and across the four asymmetric subtypes: Group 1 - mandibular body asymmetry, Group 2 - ramus asymmetry, Group 3 - atypical asymmetry and Group 4 - "C-shaped" asymmetry. *ENPP1* SNP-rs6569759 associated with asymmetry Group 1($p$=0.004), and rs858339 with asymmetry Group 3 ($p$=0.002). *ESR1* SNP-rs164321 associated with asymmetry Group 4 ($p$=0.019). These results are confirmed by Principal Component Analysis (PCA) that showed three principal components explaining almost 80% of the variation seen in the studied group. PC1 and PC2 were associated with *ESR1* SNP-rs3020318 ($p$<0.05). Diagnoses of disc displacement with reduction, masticatory muscle myalgia and arthralgia were highly prevalent in the asymmetry groups and all had strong statistical association to *ENPP1* rs858339. The average JPF scores for asymmetric subjects before surgery (JPF=7), were significantly higher than symmetric subjects (JPF=2). Patients with asymmetry Group 3 reported the highest preoperative JPF scores and Group 2 and 3 were most likely to be cured of TMD one year after treatment.

**Conclusions—**PA cephalometrics can classify asymmetry into distinct groups; identify probability of TMD and genotype associations. Orthodontic and orthognathic treatment of facial asymmetry is very effective at eliminating TMD in most patients.


## INTRODUCTION

Genesis creating symmetry and breaking of symmetry are normal developmental biologic processes. In embryogenesis, symmetry is maintained until late gastrulation and early neurulation. The Nodal Pathway regulates signal transduction to maintain symmetric dorsal-ventral patterning of mesoderm and developing nervous system.[1] The Lefty proteins, a subclass of Transforming Growth Factor-β (TGFβ) transcription proteins, act as extracellular antagonists to Nodal. During gastrulation, Lefty disrupts symmetry in germ layers to pattern normal situs positioning of the heart, lungs and gut coiling.[2] In the harmonious development of brachial arches immediately rostral, maintenance of symmetry is particularly important for natural midline fusion between right and left sides. It is well known that many genetic and environmental factors, acting separately or in unison, may produce facial clefting or developmental jaw defects in arch development.[3] Due to the potential for congenital defects, different and more subtle influences on asymmetry might be active in normal facial development. *PITX2*, an upstream effector of Nodal and Lefty signaling pathways, has recently been identified as differentially expressed between left and right side masticatory muscles in adult humans with facial asymmetry.[4] *PITX2* is necessary

for mesoderm-derived first branchial arch structures, including masticatory muscles, jaw bones and ectoderm induction for tooth formation.[5–6] Therefore, it is likely that early developmental events persist into maturation, making asymmetry an etiologic and diagnostic challenge for clinical orthodontics.[7]

Craniofacial asymmetry may be considered as a type of dentofacial deformity, which arises during facial maturation at the same time sagittal and vertical jaw discrepancies become clinically evident. The association of skeletal asymmetry with Class II or Class III skeletal malocclusion varies with the type of human population being treated, ranging from 8-50%, indicating genetic influences from gastrulation to maturation play an important role in etiology.[8–9] Skeletal malocclusions are complex trait conditions, developing from an interplay of genetic and functional (environmental) influences[10]; and new findings point toward a similar etiology for facial asymmetry.[11] In order to identify genetic contributions to skeletal malocclusion, a relatively precise description of phenotype and phenotypic variations is necessary. In orthodontics, there have been many approaches for classification of skeletal asymmetry utilizing either posterior anterior cephalograms or submentovortex radiographs; however, there is not a universally accepted method or classification system. Most recently, cone beam computed tomographic imaging has been utilized to more accurately describe and subclassify types of craniofacial asymmetry in dentofacial deformity populations.[12–13] The classification system of Baek has identified four types of asymmetry with computed tomography, which arise from different growth imbalances of the jaws, teeth, nasal septum and cranial base.[14] These new classification systems help remove much of the previous diagnostic uncertainty, since asymmetry can be masked by variations in head posture, canting of the occlusal plane or other dental and soft tissue compensations.[14–15] This etiologic – based phenotypic classification may be useful in identifying gene single nucleotide polymorphisms (SNPs) associated with asymmetry.

We have been investigating how genetic variations associate with the development of skeletal malocclusions in the patient population undergoing orthognathic surgery at the University of XXXXX. This is a typical population of French patients, relatively young with a normal distribution of open, deep, Class II and Class III skeletal malocclusions. In our population and in other studies of dentofacial deformity patients, pain in the temporomandibular joint or masticatory muscles is a common and often debilitating comorbid condition[16] estimated to occur in over 50% of patients with facial asymmetry.[17] The Baek classification might also be useful in identifying an association between the subclasses of asymmetry and prevalence of TMD.

In previous studies, we identified significant associations between SNPs in *ACTN3* (α-actinin 3) and *ESR1* (estrogen receptor α) with Class II malocclusion and *ENPP1* (ectonucleotide pyrophosphatase/phosphodiesterase 1) with mandibular prognathism.[10,18] We also found differences in gene expression for *PITX1 and PITX2* between left and right masseter muscle in asymmetry.[4] In this study, we explore associations between these genes and subclassifications of asymmetry. We also sought to determine if the subclassifications are related to the prevalence or severity of TMD before and after orthodontic treatment with jaw osteotomy.

## METHODS

### Patient Population

174 patients undergoing comprehensive orthodontic treatment with mandibular or mandibular and maxillary osteotomies for correction of jaw deformation malocclusion were recruited for study participation during their pre-surgical consultation at the University of XXXXX Department of Oral and Maxillofacial Surgery. At that time, the patients signed consent for participation according to human subject research protocols approved by the Committee for Personal Protection, University of xxxxx and the Institutional Review Board Committees at the University of XXXXXXXXX and XXXXXX University. Patients provided a saliva sample collected in Oragene Kits for genotyping. Deidentified demographic information, panorex, lateral and posterior anterior cephalograms, and clinical examination information were compiled for analysis. The patient population had a mean age of 25.7 years, majority female (76%), and a normal mixture of sagittal (66% Class II, 33% Class III) and vertical jaw deformations (75% open bite, 25% deep bite). Sagittal and vertical malocclusion classifications were based on the Delaire Cephalometric Analysis of lateral cephalograms, which is particularly useful in planning the type of osteotomy repositioning necessary to correct malocclusion.[19]

### Assessment of Asymmetry

Skeletal asymmetry was classified into four groups based on a new system adapted from Baek et. al 2012: Group 1 – lateralization of mandibular body only ("mandibular body asymmetry"); Group 2 – difference in ramus heights with menton deviation to the shorter ramus side ("ramus asymmetry"); Group 3 – difference in ramus heights with menton deviation to the longer ramus side, gonion contour more prominent on larger mandibular side and reverse maxillary canting ("atypical asymmetry"); Group 4 – difference in ramus heights with menton deviation to short ramus side and severe maxillary canting ("C-shaped asymmetry") (Figure 1). Since this classification system was derived from CBCT images of orthognathic patients, we developed a PA (posterior anterior) cephalometric analysis, which allowed us to perform comparable measurements using digital two-dimensional images with Dolphin morphometric software.

Six cephalometric measurements were used: occlusal plane tilt, maxillary canting (JR or JL to ZR or ZL), menton deviation (A to Me to MSR), mandibular width to midsagittal plane (AG or GA to MSR), mandibular width to menton (AG or GA to Me), and ramal height (Table 1 and 2). In the maxilla, occlusal plane tilt was determined by the difference between Frankfort Horizontal and the horizontal line bisecting the buccal cusp tips of UR6 and LR6, as well as the buccal cusp tips of UL6 and LL6, measured in degrees (°). If the occlusal plane tilt was greater than 2°, the subject was considered to have maxillary canting. To further verify the maxillary canting was skeletal in etiology, left and right vertical distances from jugal process and frontozygomatic suture were compared. If the difference between the left and the right side was greater than 3 mm, the subject was considered to have maxillary canting. In the mandible, menton deviation was determined by the angle between midsagittal plane and the line connecting ANS and menton. If the angle was greater than 2°, the subject was considered to have mandibular facial asymmetry with menton deviation. To compare the

left and right mandibular width, the distance between antegonial notch and midsagittal plane was compared to the contralateral side. If the difference was greater than 2 mm, it was considered to have mandibular deviation. We also measured the distance between antegonial notch and menton, and compared it with its' contralateral side. Again, if the difference was greater than 2mm, it was considered to have mandibular body asymmetry. Lastly, ramal height of left and right side was compared. If the difference between left and right side was greater than 3 mm, ramus asymmetry was diagnosed.

Subjects were diagnosed as symmetric if there was no maxillary canting (<2°), no menton deviation (<2°), and no significant difference in ramal height (<3 mm). Asymmetric subjects were further divided into four groups. Group 1 subjects had menton deviation greater than 2○ without any maxillary canting or any significant ramal height difference. Group 2 subjects had menton deviation greater than 2° with shorter ramal height on the deviated side. Group 3 subjects displayed "atypical symmetry", with shorter ramal height on the opposite of the deviated side, with slight maxillary canting towards or opposite of the deviated side. Group 4 subjects showed both shorter ramal height and maxillary canting towards the deviated side, displaying "C-shaped asymmetry" as described by Baek et al.[14] Furthermore, principal components analysis (PCA) explaining more than 5% of the facial skeletal variation were selected for genotype-phenotype correlation analysis. Data were normalized and standardized using a linear model to assess the possible effects of age and sex and to consider the possibility of age-by-sex interactions.

### Genotype Assessment

Saliva samples, one per patient, were collected in Oragene kits and prepared for DNA extraction and posterior genotyping using TaqMan chemistry and end-point analysis in an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems, Foster City, CA), as described previously.[10] Thirteen SNPs were selected for genotyping: in *ACTN3* rs1815739, and rs678397[10]; in *ENPP1* rs937300, rs6569759, rs858339 and rs1409181[18,20–22]; in *ESR1* rs1643821, rs302318, rs3020377 and rs2077647[18,23–26]; in *PITX1* rs1131611 and in *PITX2* rs2595110[4] to determine if specific allelic variants are over-represented in subjects with malocclusion sub-classifications. Thirty-three additional anonym SNPs were genotyped.

### Assessment of TMD

TMD was assessed using the routine clinical examination done by the Maxillofacial Surgeons before surgical treatment and entered into the Research Diagnostic Criteria for Temporomandibular Disorders (DC/TMD).[27] In addition, the Jaw Pain and Function (JPF) questionnaire was used to determine the presence and severity of TMD, as a subjective patient report. The JPF Questionnaire was developed as a screening tool to determine presence or absence of TMD conditions.[28] It consists of 8 questions relating to jaw pain and 5 questions related to jaw function. The questionnaire has been validated to reliably distinguish between normal and TMD subjects with up to 98% sensitivity and 100% specificity when a cut off score of 6 is used for responses.[29] The questionnaire has been translated for use in Germanic,[30] and we prepared a French version for use in Lille, as a standard assessment of presence and severity of TMD.[18]

One hundred twenty one patients have attended a one year post-treatment reevaluation appointment, where they completed a second JPF survey. Patients were divided into five groups for comparative purposes based upon difference in pre-surgical and one year post-treatment JPF scores. Patients were classified in the following five groups: *No Change* in TMD, *Improvement* of TMD if the JPF score decreased by 3 or more; *Worsening* of TMD if the JPF score increased by 3 or more; *Cured* of TMD if the JPF score was > 6 before surgery and < 6 after treatment; and *Iatrogenic* TMD if the JPF score was < 6 before surgery and > 6 after treatment. We used the terms "*cured*" or "*iatrogenic*" since a score > 6 is diagnostic for presence or absence of TMD with the JPF assessment.

**Statistical Analysis—**For cephalometric assessment of asymmetry, an unpaired t test was used to compare if differences between sides for individual cephalometric measurements were significantly different between symmetric and asymmetric patients. An ANOVA test was used between the four subclassifications of asymmetry to determine anatomical differences between groups. Tests for measurement error included intrarater reliability in cephalometric measurements (by repeating cephalometric tracing on 10% of the radiographs by 1 examiner [K.C.]; this resulted in an $R^2$ value of 0.98.

For genotype assessment, the characteristics of the population were presented with the usual rules of descriptive statistics: frequencies and percentages for categorical variables; mean and standard deviation for quantitative variables. The association between the change in JPF scores, TMD diagnosis and clinical or surgical characteristics of the population was assessed by Chi-square test for categorical variables or Fisher exact test in the case of small numbers. Quantitative variables were analyzed using Student's t-test. When the distribution of the variable did not follow a normal distribution, a nonparametric Wilcoxon test was performed. After ensuring compliance with the Hardy-Weinberg equilibrium, the association of different SNPs was also sought by a Chi-square test or Fisher exact test when small numbers were present. For each SNP, the analysis was performed by considering the three genotypes separately, as well as by calculating the total number of alleles.

For PCA, SNPs were coded 0, 1, and 2 according to the number of minor allele copies. Multivariate linear regressions adjusting for age, sex, and ethnicity were performed to test for associations between each SNP (one at a time) and the selected principal components. The same Bonferroni threshold described above was used here. All analyses were performed with SPSS software for Windows (version 20.0; IBM, Armonk, NY).

For TMD assessment, an unpaired t-test was used to determine if there were significant differences in pre-surgical JPF scores between symmetric and asymmetric patients, and an ANOVA test was performed between the four asymmetric groups. An ANOVA was also used to compare if there were significant changes in the JPF score 1 year after treatment between the symmetric group and the four asymmetric groups. To further evaluate, a post-hoc t-test was done to compare individual JPF scores of asymmetric groups.

## RESULTS

The patient population represented a normal demographic distribution of subjects seeking orthodontic and orthognathic surgery treatment of dentofacial deformity malocclusion from the geographic area of Northern XXXXXX and Southern XXXXXXX. All patients treated for the condition were referred to the Oral and Maxillofacial Surgery Department at the University of xxxxx under the National Health Care Service of France. 52% of patients were diagnosed as symmetric and 48% were diagnosed as asymmetric. Segregating the asymmetric subjects into the four proposed subtypes was relatively easy to accomplish utilizing 11 cephalometric anatomic landmarks and 6 cephalometric measurements (Table 1 and Table 2, Figure 1). This classification system was validated by the very significant differences obtained by comparing the cephalometric measurements between symmetric and asymmetric groups, and asymmetric subtypes (Table 3). All six PA cephalometric measurements were compared between symmetric and asymmetric patients, and across the four asymmetry subclassification groups. For bilateral points, values are the difference between the left and the right side. The PA cephalometric analysis results demonstrated a significant difference in all six cephalometric measurements between symmetric and asymmetric patients. All mandibular measurements, such as menton deviation, mandibular width (GA or AG to MSR and GA or AG to Menton), and ramal height showed notably significant differences between symmetric and asymmetric patients, clearly indicating the presence of mandibular asymmetry.

Cephalometric measurements were also compared across different asymmetry groups (Figure 2). Generally, asymmetry group 4 showed a greater amount of canting and deviation when comparing cephalometric measurements. It had the most severe malformations with an occlusal plane tilt, maxillary canting and mandibular deviation that were proportionally much more imbalanced than in other asymmetry groups. Because group 4 had maxillary canting, the ANS to menton line used to measure the degree of chin deviation in the mandible was higher than in the other three groups $p = 0.003$ (Figure 2). The second characteristic evident with asymmetry was a worsening of ramal height differences from group 1 to group 4. Differences in PA Cephalometric measurements indicate the four subtypes may be considered as anatomically different forms of asymmetry that can be compared for differences in genotype variations within the study. We therefore compared differences in genotype for symmetric and asymmetric subjects and between asymmetric subtypes (Table 4).

Significant differences were detected for *ENPP1* and *ESR1* genotypes, but not for *ACTN3*, *PITX1* and *PITX2*. *ENPP1* SNP rs6569759 was significantly different for genotype and alleles for group 1 compared to other asymmetry subtypes. *ENPP1* SNP rs858339 was different for genotype between symmetric and asymmetric groups and for group 3 compared to other asymmetric groups. *ESR1* SNP rs1643821 was also significantly different for genotype and allele for asymmetry group 4 compared to the other asymmetric groups. Individuals with group 1 asymmetry were almost four times more likely to carry the G allele of *ENPP1* rs6569759 (OR=3.89; 95% confidence intervals 1.02–14.78). These results further support the appropriateness of the asymmetry subclassifications, as this phenotypic organization can be utilized to recognize meaningful genotypic differences.

Principal Component analysis (PCA) disclosed three principal components (PC1 to PC3) explaining more than 5% of the shape variation in the asymmetric subjects. The cumulative variation score of each component totalized 79.768% of the subject's variability (Table 5). (Supplemental table 1). PC1 comprised 39.721% of the sample variability and showed asymmetric individuals with low values to occlusal plane tilt angle and high values to maxillary canting. PC2 comprised 23.976% and disclosed low scores to occlusal plane tilt and mandibular width to midsagittal Plane (GA-MSR) and high scores to menton deviation (A-Me-MSR). PC3 showed 16.070% of shape variability and disclosed low scores to mandibular width to menton and high scores to ramal height. Regarding PCA and the subjects genotype, PC1 and PC2 were associated to SNP in *ESR1* (rs3020318) ($p$=0.04). (Supplemental table 2). To determine how variation in principle components related to PA cephalometric measurements we used the Varimax with Kaiser Normalization rotation method to plot components in rotated space. Variance in total cephalometric measurements for each symmetric or asymmetric patient was visualized by a component plot rotated in space (Figure 3). A second component plot visualized the variance in each individual cephalometric measurement for all patients (Figure 4).

Since TMD is often present in this patient population before initiation of treatment, we wanted to know if there was a higher prevalence within and between asymmetry subclassifications. TMD diagnosis was positive in only 3% of symmetric patients and very high in asymmetric (Table 5). Disc displacement with reduction was most common in asymmetric patients (78%), followed by 61% with myalgia of masticatory muscles, 33% with arthralgia and 12% with TMD-related headache. Disc displacement without reduction was the least common Axis I diagnosis at 6%. Overall the population is young, with an average age of 26 years and not presenting with fibromyalgia or pain related disability diagnosed in Axis II of the diagnostic criteria. Comparing genotypes to TMD diagnosis for disc displacement with reduction, myalgia and arthralgia, and not disc displacement without reduction or TMD-related headache since these groups have very few individuals; *ENPP1* rs858339 had significant associations for genotype and/or allele for all three TMD diagnosis (Table 6).

We utilized the JPF questionnaire as an efficient assessment tool for patient perception of presence and severity of TMD. Most symmetric subjects had little to no symptoms for TMD and an average JPF score of 1.97, which agreed with the clinical exam assessment for TMD (Table 7). Asymmetric subjects had an average JPF score of 6.87, which was significantly higher than in symmetric. Further, there were significant differences within asymmetric subtypes with Group 3 having the highest average score of 9.11 and Group 1 and 4 having the lowest average scores of about 4. Comparing JPF scores between groups, a t-test demonstrated very significant differences between symmetric and asymmetric patients $p<0.001$. Within the asymmetric patients an ANOVA comparison also revealed very significant differences between subclassifications. These results indicate there is more likely a chance for signs or symptoms of TMD when asymmetry is part of a patient's dentofacial deformity.

After treatment, we followed the subjects for one year to determine if TMD improved, worsened or if new conditions occurred. Overall, those without TMD remained so, with 52%

of the patients having no change in JPF score (Figure 5). Twenty percent of all patients were cured of TMD with post treatment JPF scores below 6 and 17% had an improvement with JPF scores decreased by 3 or more. Four percent of the patients had worsening of TMD after treatment with JPF scores increased by 3 or more. Finally, 7% could be diagnosed with occurrence of TMD with JPF scores < 6 before surgery and > 6 after treatment. All groups, including the symmetric group, had decrease in post-treatment JPF scores that were statistically significant by ANOVA comparison, $p<0.001$. Further post hoc t-tests revealed significantly greater decreases in post-treatment JPF scores for asymmetry group 2 and 3 compared to the symmetric group, $p<0.001$.

## DISCUSSION

Craniofacial asymmetry, which arises from normal developmental processes, takes many shapes and forms, given the complexity of the nervous, skeletal, muscular and physiologic components that drive cognitive, sensory, stomatognathic and respiratory functions.[32] A functional variation or genetic polymorphism influencing an individual or multiple components can lead to a localized skeletal asymmetry, or a generalized craniofacial asymmetry depending upon the cause and growth response over time.[33] Lateral skeletal distortions are known to occur in the skull,[34] cranial base,[35–36] midfacial structures, including the vomer, pterygoid process, piriform apertures and maxilla,[4] and in the mandible by hemimandibular elongation or hyperplasia.[37] These ubiquitous configurations of asymmetry have made simple classifications for patterns of craniofacial growth or etiology elusive. Yet a systematic classification system is needed to more accurately plan surgical corrections[14,38] and serve as a phenotypic clustering for gene association studies.[39] Using either traditional tracings of PA cephalograms[40] or cluster analysis of 3-dimensional computed tomographic image analysis, a classification system with 4 groups or subtypes of craniofacial asymmetry have been formulated for Korean people.[14] Our first objective was to determine if French people with asymmetry could also be subclassified using this diagnostic grouping. We were able to adapt the asymmetry classification system of Baek et al.[14], which used 3-dimensional computed tomography, with a new cephalometric analysis of posterior anterior cephalograms for our patients. The diagnostic classification identified differences between groups due to the significant differences in cephalometric measurements. However, the French population differed from the Korean population in the percent distribution amongst the asymmetric groups. Koreans were most likely to have Group 1 or Group 2 asymmetry while the French were most likely to have Group 2 or 3. The French patients were almost equally matched with 52% of subjects undergoing orthognathic surgery being symmetric and 48% being asymmetric. To our knowledge, no similar estimates are available for Koreans, but in Singapore the prevalence of asymmetry ranges from 8 to 50% in orthognathic surgery patients.[40] These population differences most likely stem from three distinct influences. The first is the tendency for the percentage of asymmetry to differ in different combinations of Class II, III, open and deep bite malocclusions.[41] The second is the almost certain likelihood that genetic differences in facial shape that mark race and ethnicity can also influence the distribution of asymmetry subclassifications.[4] Finally, there were differences on the type of images being utilized in diagnosis, and some differences in the morphology of asymmetry sub-classification groups.

### Posterior Anterior Cephalometric Measurement Errors

Although PA cephalometric analysis has been the traditional approach for diagnosis of asymmetry, variability of head positioning in the cephalostat may introduce radiographic projection errors, which diminish diagnostic reliability.[42] Cone-beam computed tomography (CBCT) images are considered more reliable for diagnosis of asymmetry, but there is insufficient evidence to conclude that CBCT is superior to PA celphalgrams for detecting transverse facial differences.[43] We routinely use PA cephalograms in surgical treatment planning of patients and attempt to minimize head rotation around the vertical Z-axis where the majority of projection error occurs[44]. A second potential error is landmark identification due to unclear radiographic representation of anatomical locations. However, the four patterns of asymmetry are almost always discernible by visual observation, which helps decrease uncertainty as to where landmarks are located. There have been limited reports on landmark identification on PA cephalograms[45], but a recent report (Ulkur et al., 2016[46]) estimates rater reliability to be consistently high at 0.9 to 0.95, or above for most points. Our intrarater reliability had an $R^2$ value of 0.98, and is similar to Ulkur. Therefore our methods may introduce some measurement error in diagnosis, but given the large differences in specific cephalometric measures between groups (Figure 2), was not a major influence on classification of subjects into asymmetry groups.

### Genotype Associations

The second study objective was to determine if gene variations already identified as contributing to sagittal and vertical malocclusion (*ACTN3*, *ENPP1*, ESR1, *PITX1* and *PITX2*) might also associate with asymmetry (Table 4). We compared 12 SNPs to determine differences for genotype and allele between symmetric vs. asymmetric patients and between the asymmetric groups. Two SNPs were associated with differences in both genotype and alleles when compared between asymmetry groups. SNP rs6569759 in *ENPP1* was different in group 1 and SNP rs1643821 in *ESR1* was significantly different in group 4. The SNP rs858339 in *ENPP1* was very significantly different for genotype between symmetric vs. asymmetric subjects and between asymmetric groups for group 3.

*ENPP1* has pleotropic effects for mineralization and insulin signaling. The intronic SNP rs6569759 has previously be associated with an increased risk for type 2 diabetes, which results insulin-mediated glucose metabolism, which affects fiber type composition of skeletal muscles.[47] The SNP has also been associated to changes in bigonial width dimension in Western Eurasians.[48] This corroborates our finding that rs6560759 is significantly different in group 1, which has asymmetry in mandibular body breadth, but not in ramus height. Rs858339 is an intronic SNP previously associated with variation in bone mineral density.[49] We have recently reported that rs858339 has a significant association with the occurrence of preoperative TMD in our patient population.[18] *ENPP1* rs858339 TT genotype associated with pretreatment absence of TMD and the AT genotype as a pretreatment risk factor for presence of TMD as determined by JPF scores.[18] In this study rs858399 has strong statistical association with the TMD diagnoses of disc displacement with reduction, masticatory muscle myalgia or arthralgia, further confirming its role in etiology (Table 5). The association of *ENPP1* to presence of TMD diagnoses or asymmetry group 3 is not yet informative as to the biological mechanisms responsible for occurrence.

The conditions of arthralgia and disc displacement may be related to *ENPP1* biomineralization functions and myalgia to insulin signaling in skeletal muscle, but more investigations will be necessary to confirm these possibilities.

*ESR1* polymorphisms associate with skeletal Class II malocclusions and symptomatic osteoarthritis of the TMJ in Korean women.[50] The rs1643821 intron SNP contributes to the susceptibility for osteoporotic fracture in postmenopausal Chinese women.[51] Since the incidence of TMD in asymmetry group 4 is relatively low, rs1643821 may be contributing to development of this type of asymmetry through variations in bone mineral density with growth, rather than by specific problems in the TMJ.

We used PCA as a secondary procedure for analysis of PA cephalometric measurements. Three components explained 80% of morphologic variation found from cephalometric analysis. Principal component (PC) one identified variability related to occlusal plane tilt and maxillary canting, component two to mandibular width and menton deviation and component three to ramal height (Supplemental table 1). A component plot rotated in space demonstrated a clustering of symmetric patients for overall variance in cephalometric measurements that was separated from asymmetric patients (Figure 3). Asymmetric patients also had greater variability in morphologic variation. A second component plot summarized how the variation in individual cephalometric measurements identified in Figure 2 related to each other morphologically. Left and right ramal height and left and right maxillary canting (JL-ZL with JR-ZR) clustered together, indicating that variation in these measures was similar between the left and right face. Two opposite measures also clustered together: AG-Menton with GA-MSR and GA-Menton with AG-MSR; indicating that menton deviation on one side of the face matched variance in mandibular body width on the opposite side. Variation in occlusal plane tilt was an independent morphologic entity which had limited relationship to other component variations. The relationship of ANS-Me-MSR was a second independent morphologic entity, which reflected the independent variation of nasal septal deviation relative to other facial structures.

Genotype analysis associated SNP rs3020318 in *ESR1* with principal components one and two. (Supplemental table 2). Although not directly comparable to the genotype associations for the four asymmetry groups, the PCA analysis provided a confirmatory finding. *ESR1* rs1643821 associates with asymmetry group 4 and rs3020318 with PC one and two. PC one and two relate to transverse canting and menton deviation respectively, and both of these morphologic features are found in group 4. rs3020318 is an intronic SNP listed as without any known functional effects. However, this marker belongs to a haplotype associated with greater cancer risk.[52] There is growing evidence that the impact of genetic risk factors on breast cancer varies by hormone receptor status. Genetic variation of the estrogen metabolism pathway -particularly the genes involved in the production of estrogen through androgen conversion -influences the risk for the development of estrogen-sensitive breast cancer.[53] This could be also true for craniofacial deformities that include symptomatology in the temporomandibular joint, with variation in hormonal levels (*i.e.* estrogen) influences the risk for development of TMD.

While there is not a specific polymorphism associated with *PITX2* and facial asymmetry, differences in gene expression were previously identified in right and left masseter muscle samples from asymmetric patients[4]. The variation in *PITX2* gene expression likely has an association with facial asymmetry, and warrants further research. One possibility to be investigated is that *PITX2* may interact with *ENPP1* to produce differences in mineral density and bone growth between facial sides.

### Asymmetry and pre- or post-operative TMD

There is broad consensus among orthodontists that little to no relationship exists between specific types of malocclusions and development of TMD.[54] Yet for many people with jaw deformations, pain in the TMJ or masticatory muscles is a common and debilitating comorbid condition estimated to occur in over 50% of patients with facial asymmetry.[16–17] Our results affirm this finding. The JPF scores for asymmetric subjects were significantly higher than symmetric subjects ($p>0.0001$). Further, asymmetry groups 2 and 3 were most likely to have pre-operative TMD present before orthodontic treatment was initiated. In order to determine if the musculoskeletal imbalances were the root cause of TMD, we followed these patients at least one year after orthodontic treatment to see if the condition resolved or persisted. Group 2 and 3 were most likely to be cured of TMD at one year follow-up or at least had significant symptom improvement (Figure 5). There is growing evidence, confirmed on different patient populations that dentofacial deformities have higher prevalence of TMD,[55] and when treated with combined orthodontic and surgical procedures, usually have better masticatory function and improved TMD symptoms, especially for the relief of pain[56–57].

These findings lead to the conclusion that orthodontic and orthognathic treatment of craniofacial asymmetry helps cure or alleviate TMD for the majority of patients. However, as with any clinical treatments there were a small percentage of patients who had significant worsening or presentation of TMD symptoms in the retention phase (7%). Most of these (80%) were subjects without asymmetry, who we would not have expected this to occur. Other post-treatment conditions, such as condylar remodeling or condylar resorption could be influencing this rare TMD occurrence and will be studied in the future. Do to the small number of subjects with this result, 5 total, many more patients will need to be followed for further characterization of post-treatment development of TMD. Overall, these treatments are highly effective at correcting skeletal malocclusions, producing a physiologic balance that alleviates TMD symptoms.

## CONCLUSIONS

1. A new posterior anterior cephalometric analysis utilizing six measurements to detect differences in facial sides has been developed to distinguish four main classifications of asymmetry that are common in dentofacial deformitiy patients.
2. TMD prevalence is much higher in patients with asymmetry compared to dentofacial deformity patients without asymmetry. The most common TMD presentations were disc displacement with reduction, masticatory muscle

myalgia and arthralgia. Two of the four asymmetry groups had both high positive diagnosis for TMD and subjective patient reporting of symptom.

3. SNP genotype rs6569759 in *ENPP1* associated with asymmetry group one and rs858339 with asymmetry group three.
4. SNP genotype rs1643821 in *ESR1* associated with asymmetry group four. rs3020318 in *ESR1* associated with PC one and two, which relate to maxillary canting and menton deviation.
5. SNP genotype rs858339 in *ENPP1* associated with presence of disc displacement with reduction, masticatory muscle myalgia and arthralgia.
6. Orthodontic and orthognathic treatment of asymmetry elevates TMD symptoms for at least one year into retention in most of the patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Highlights for review**

1. A new asymmetry classification system utilizing PA cephalograms, producing 4 diagnostic groups.
2. Pre-treatment TMD is elevated in some of the asymmetric diagnostic groups.
3. *ENPP1* and *ESR1* genotypes and alleles associate with 3 of the 4 asymmetry groups.
4. Orthodontic and orthognathic treatment of asymmetry elevates TMD symptoms for at least one year into retention in most patients.
5. New insights into what types of asymmetry predispose to TMD; a basis for future studies.

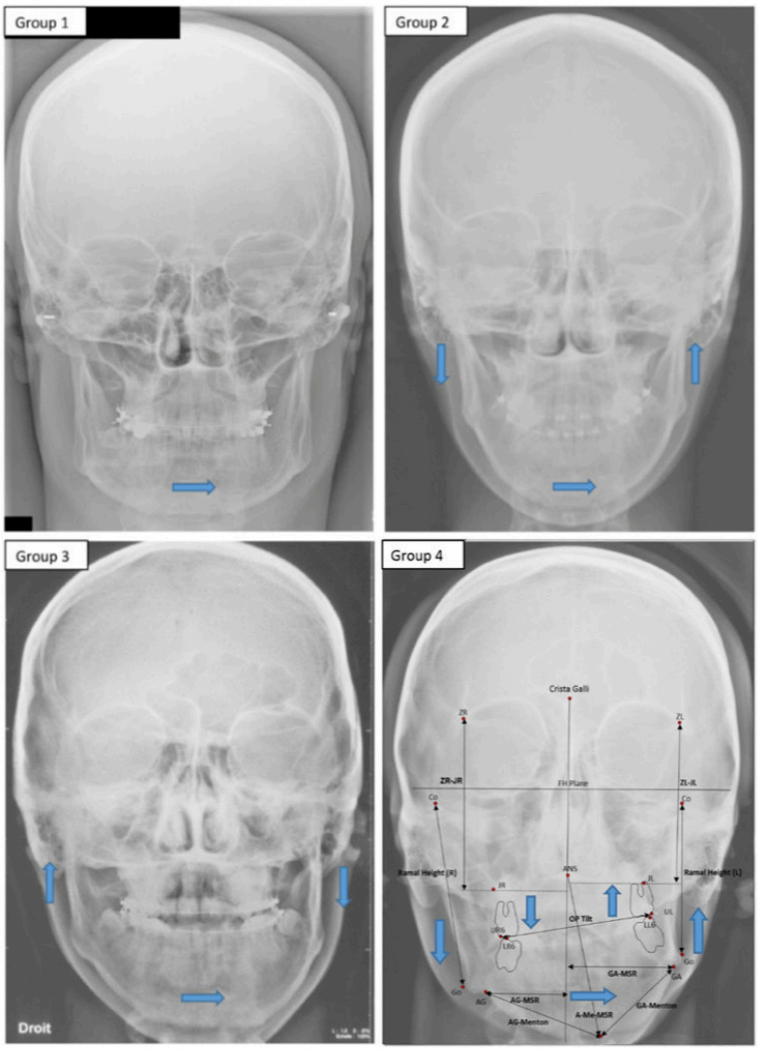


**Figure 1. Prototypes for four asymmetry subtypes and illustration of PA cephalometric tracing**
Group 1 - mandibular body asymmetry, Group 2 - ramus asymmetry, Group 3 -atypical asymmetry, and Group 4 - C-shaped asymmetry. Landmarks used for cephalometric analysis labeled on Group 4.

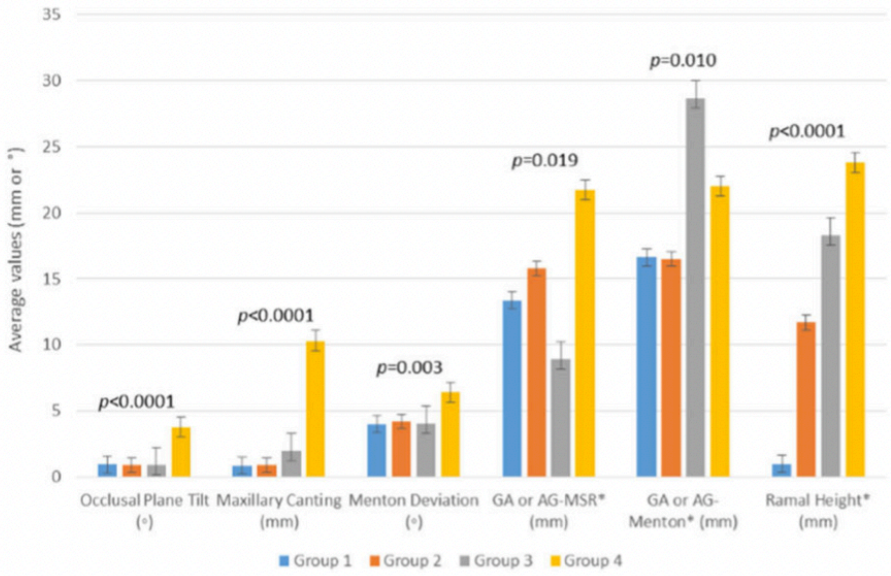


**Figure 2. Histogram of cephalometric measurement comparisons by different asymmetry groups**

Average values for each cephalometric measurement were compared across different asymmetry groups. *For bilateral points, the difference between left and right side was used and denoted as a positive value. Average values are expressed in mm for maxillary canting, GA or AG-MSR, GAor AG-Menton and ramal height, and degrees for occlusal plane tilt and menton deviation.

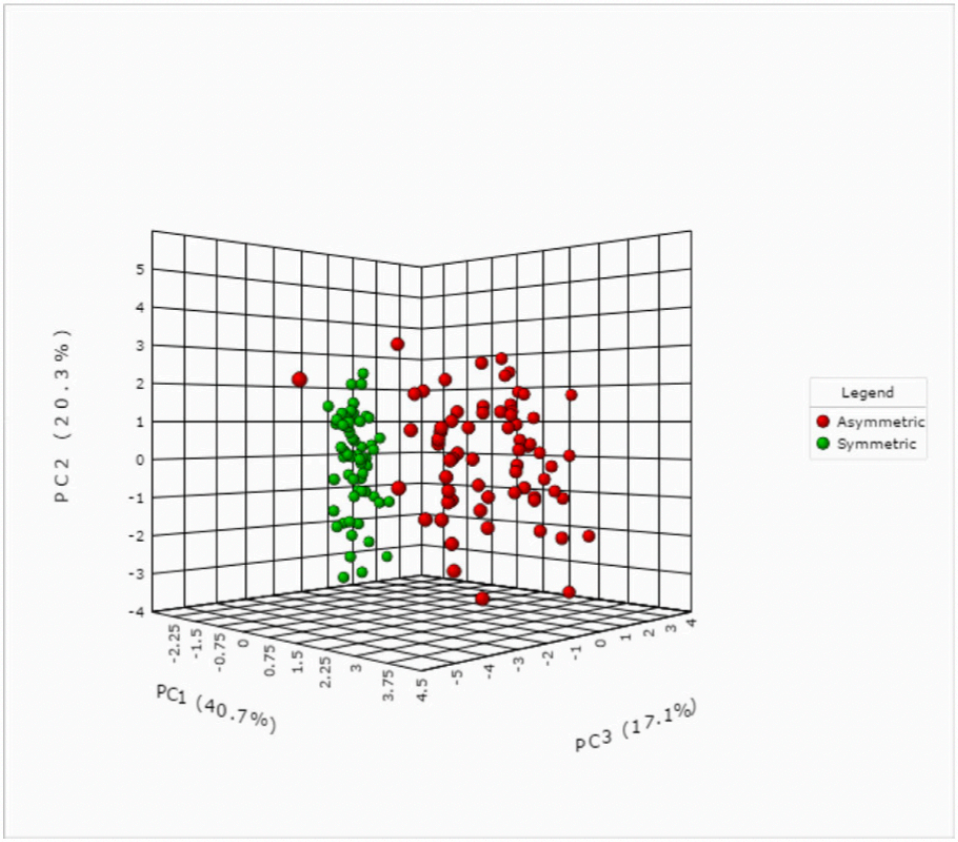


**Figure 3.**
A Component Plot in Rotated Space representing variation in all cephalometric measurements of individual patients, classified as either symmetric or asymmetric, and their interrelationships for Principal Components one to three.

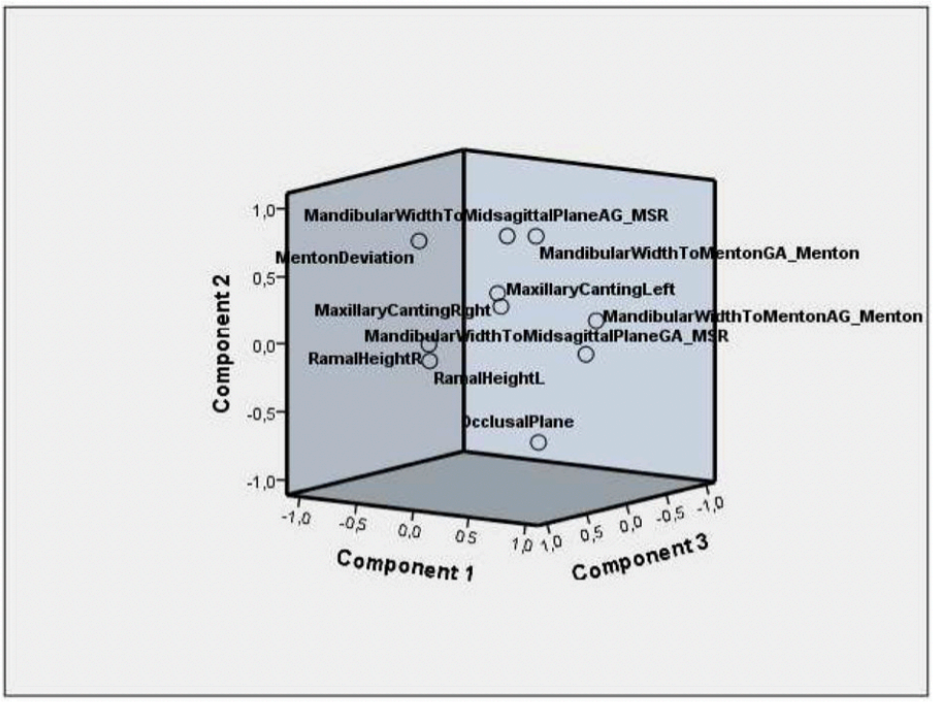


**Figure 4.**
A Component Plot in Rotated Space representing variation in individual cephalometric measurements and their interrelationships for Principal Components one to three.

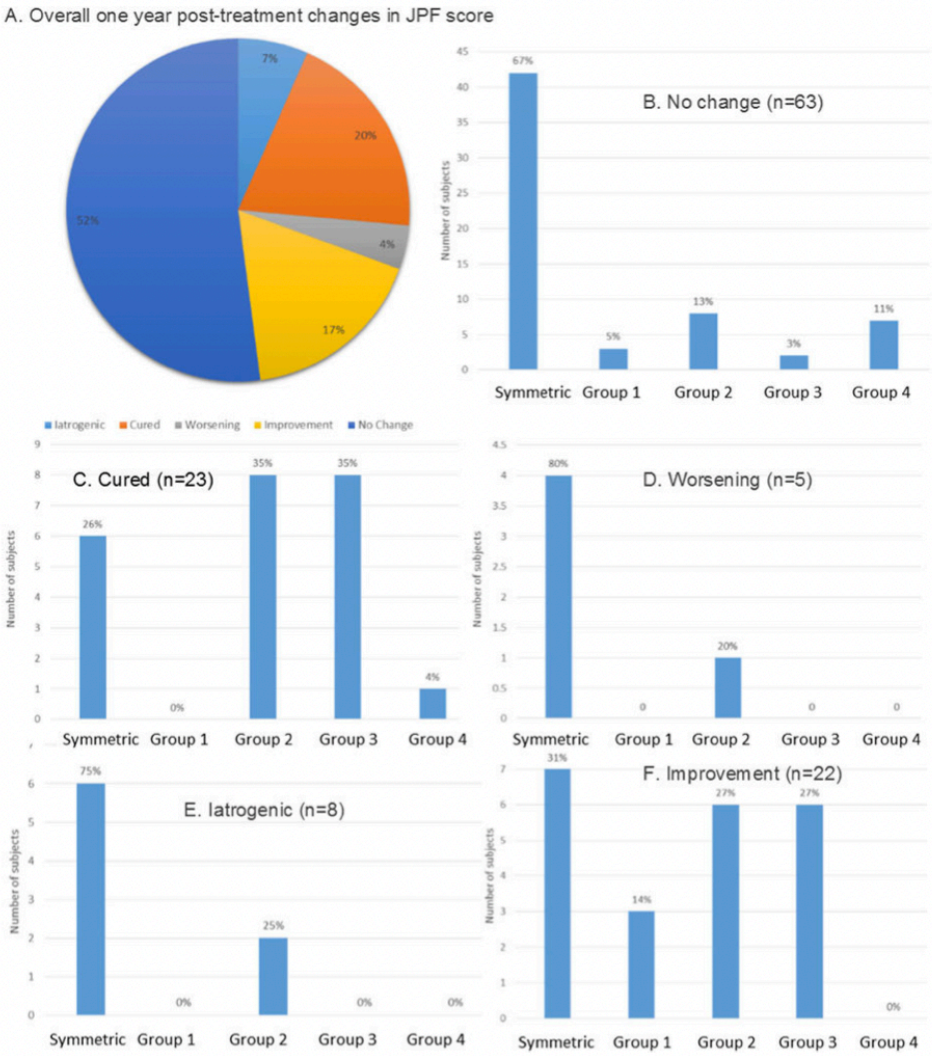


**Figure 5. One year post-treatment changes in JPF score by asymmetry classification**

**A. Distribution of patients based on difference in pre-surgical and one year post-surgical JPF scores:** 1) No Change in TMD, 2) Improvement of TMD if the JPF score decreased by 3 or more; 3) Worsening of TMD if the JPF score increased by 3 or more; 4) Cured of TMD if the JPF score was > 6 before surgery and < 6 after treatment; and 5) Iatrogenic TMD if the JPF score was < 6 before surgery and > 6 after treatment. **B. Distribution of symmetric and asymmetric groups in "No Change" category. C. Distribution of symmetric and asymmetric groups in "Cured" category. D. Distribution**

**of symmetric and asymmetric groups in "Worsening" category. E. Distribution of symmetric and asymmetric groups in "Iatrogenic" category. F. Distribution of symmetric and asymmetric groups in "Improvement" category**.

**Table 1**

Lndmarks used for PA cephalometric analysis

| Landmarks | Definitions |
|---|---|
| ANS | Anterior nasal spine |
| AG/GA | The highest point in the antegonial notch (left and right) |
| Co | Condylion; Most superior point on condylar head |
| Crista Galli | Most superior point at its intersection with the sphenoid |
| Gonion/Most Lateral Ramus | Most inferior, posterior, and lateral point at the gonial angle of the mandible |
| JL/JR | Bilateral points on the jugal process at the intersection of the outline of the tuberosity of the maxilla and zygomatic buttress |
| Me | Menton; most inferior point at symphysis |
| Midsagittal Plane | A plane bisecting the head and face through the crista galli, ANS, and genial tubercles in a symmetric face |
| Occlusal Plane | Horizontal line bisecting UR6 and LR6 as well as UL6 and LL6 |
| UR6/UL6<br>LR6/LL6 | Buccal cusp tip of right/left maxillary molar<br>Buccal cusp tip of right/left mandibular molar |
| ZL/ZR | Medial aspect of frontozygomatic suture (Bilateral) |

**Table 2**

Measurements used for evaluation of facial asymmetry in maxilla and mandible

| Measurements | Definitions |
|---|---|
| Maxilla: | |
| Occlusal Plane Tilt (○) | Difference between Frankfort Horizontal and the horizontal line bisecting UR6 and LR6 as well as UL6 and LL6 |
| Maxillary Canting JL or JR – ZL or ZR (mm) | Difference between vertical distance from left or right jugal process between left or right frontozygomatic suture |
| Mandible: | |
| Menton Deviation A-Me-MSR (○) | Angle formed between midsagittal plane and line going though ANS and menton |
| Mandibular Width to Midsagittal Plane GA or AG-MSR (mm) | Distance between left or right antegonial notch and midsagittal plane |
| Mandibular Width to Menton GA or AG-Menton (mm) | Distance between left or right antegonial notch and menton |
| Ramal Height (R or L) (mm) | Linear distance between condylion to most lateral ramus (gonion) |

**Table 3**

Cephalometric measurement comparison between symmetric and asymmetric subjects and statistical

| Measurements | Symmetric | Asymmetric | *P* value* |
|---|---|---|---|
| Occlusal Plane Tilt (○) | 0.87 ± 0.59 | 1.36 ± 1.47 | 0.0040 |
| Maxillary Canting JL or JR – ZL or ZR (mm) | 0.68 ± 0.44 | 2.70 ± 5.17 | 0.0003 |
| Menton Deviation A-Me-MSR (○) | 0.31 ± 0.29 | 4.47 ± 2.10 | <0.0001 |
| Mandibular Width to Midsagittal Plane GA or AG-MSR (mm) | 1.51 ± 1.31 | 14.2 ± 12.8 | <0.0001 |
| Mandibular Width to Menton GA or AG-Menton (mm) | 1.51 ± 2.69 | 21.4 ± 14.9 | <0.0001 |
| Ramal Height (mm) significance | 1.41 ± 0.79 | 14.5 ± 12.1 | <0.0001 |

*Unpaired t-test derived from mean and SD.

**Table 4**

Comparison of SNP genotypes by symmetry subclassifications

| Gene | SNP | Genotype | Asymm-1 | Asymm-2 | Asymm-3 | Asymm-4 | Symmetric | Symmetry vs. Asymmetry | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | p value - genotype | p value - allele |
| *ENPP1* | rs9373000 | AA | 4 (57) | 17 (65) | 9 (41) | 6 (67) | 35 (54) | | |
| | | AG | 2 (29) | 7 (27) | 10 (46) | 3 (33) | 28 (43) | | |
| | | GG | 1 (14) | 2 (8) | 3 (13) | 0 (0) | 2 (3) | 0.24 | 0.83 |
| | *p* - value genotype | | 0.33 | 0.21 | 0.15 | 0.62 | | | |
| | *p* - value allele | | 0.23 | 0.46 | 0.13 | 0.19 | | | |
| | rs6569759 | GG | 5 (72) | 6 (21) | 5 (22.5) | 2 (22) | 11 (17) | | |
| | | AG | 1 (14) | 12 (46) | 2 (55) | 5 (56) | 38 (58) | | |
| | | AA | 1 (14) | 8 (33) | 5 (22.5) | 2 (22) | 16 (25) | 0.27 | 0.38 |
| | *p* - value genotype | | ***0.004*** | 0.56 | 0.83 | 0.92 | | | |
| | *p* - value allele | | ***0.016*** | 1 | 0.66 | 0.76 | | | |
| | rs858339 | TT | 1 (16) | 3 (9) | 0 (0) | 0 (0) | 20 (30) | | |
| | | AT | 3 (42) | 10 (36) | 13 (59) | 4 (44) | 12 (18) | | |
| | | AA | 3 (42) | 15 (55) | 9 (41) | 5 (56) | 33 (52) | ***0.002*** | 0.55 |
| | *p* - value genotype | | 0.33 | 0.13 | ***0.002*** | 0.33 | | | |
| | *p* - value allele | | 0.27 | 0.77 | 0.14 | 0.24 | | | |
| | rs1409181 | CC | 1 (14) | 5 (12) | 5 (22.5) | 1 (12) | 9 (15) | | |
| | | CG | 4 (57) | 15 (55) | 3 (59) | 7 (76) | 36 (55) | | |
| | | GG | 2 (29) | 8 (33) | 4 (18.5) | 1 (12) | 20 (30) | 0.53 | 0.31 |
| | *p* - value genotype | | 0.99 | 0.88 | 0.41 | 0.41 | | | |
| | *p* - value allele | | 0.92 | 0.69 | 0.21 | 0.49 | | | |
| *ESR1* | rs1643821 | GG | 1 (16) | 3 (9) | 3 (13) | 3 (32) | 4 (7) | | |
| | | AG | 3 (42) | 14 (50) | 10 (46) | 5 (56) | 36 (55) | | |
| | | AA | 3 (42) | 11 (41) | 9 (41) | 1 (12) | 25 (38) | 0.23 | 0.3 |
| | *p* - value genotype | | 0.63 | 0.72 | 0.48 | ***0.019*** | | | |
| | *p* - value allele | | 0.58 | 0.8 | 0.76 | ***0.02*** | | | |

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| Gene | SNP | Genotype | Asymm-1 | Asymm-2 | Asymm-3 | Asymm-4 | Symmetric | Symmetry vs. Asymmetry | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | p value - genotype | p value - allele |
| | rs3020318 | TT | 3 (43) | 12 (46) | 12 (55) | 1 (12) | 24 (37) | | |
| | | CT | 4 (57) | 13 (45) | 7 (32) | 7 (76) | 28 (43) | | |
| | | CC | 0 (0) | 3 (9) | 3 (13) | 1 (12) | 13 (20) | 0.39 | 0.27 |
| | *p* - value genotype | | 0.47 | 0.46 | 0.4 | 0.16 | | | |
| | *p* - value allele | | 0.17 | 0.29 | 0.23 | 0.4 | | | |
| | rs3020377 | GG | 3 (43) | 13 (45) | 12 (55) | 3 (33) | 27 (41) | | |
| | | AG | 4 (57) | 12 (46) | 6 (26.5) | 6 (67) | 31 (48) | | |
| | | AA | 0 (0) | 3 (9) | 4 (18.5) | 0 (0) | 7 (11) | 0.78 | 0.51 |
| | *p* - value genotype | | 0.65 | 0.8 | 0.23 | 0.28 | | | |
| | *p* - value allele | | 0.22 | 0.51 | 0.73 | 0.91 | | | |
| | rs2077647 | TT | 2 (29) | 2 (8) | 3 (13) | 1 (12) | 7 (11) | | |
| | | CT | 2 (29) | 20 (71) | 11 (51) | 6 (67) | 34 (52) | | |
| | | CC | 3 (42) | 6 (21) | 8 (36) | 2 (21) | 24 (37) | 0.63 | 0.53 |
| | *p* - value genotype | | 0.37 | 0.2 | 0.98 | 0.67 | | | |
| | *p* - value allele | | 0.71 | 0.52 | 0.93 | 0.59 | | | |

**Table 5**

Percentage of TMD diagnosis among classification groups

| | Symmetric (n=90) | All Asymmetric (n = 84) | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|---|---|
| DDR* | 3 % | 78 % | 11 % | 29 % | 52 % | 23 % |
| Myalgia | 4 | 61 | 11 | 33 | 44 | 23 |
| Arthralgia | 1 | 33 | 0 | 17 | 19 | 8 |
| Headache** | 0 | 12 | 0 | 9 | 7 | 8 |
| DD w/o R*** | 0 | 6 | 0 | 3 | 11 | 0 |

*DDR = Disc displacement with reduction

**Headache attributed to TMD

***DD w/o R = Disc displacement without reduction with limited opening

**Table 6**

*ENPP1* rs858339 genotypes by DC-TMD Classification

| Gene | SNP | Genotype | No TMD | DDR | Myalgia | Arthralgia |
|---|---|---|---|---|---|---|
| *ENPP1* | rs858339 | TT | 50 (67) | 9 (35) | 0 (0) | 0 (0) |
| | | AT | 21 (28) | 16 (62) | 14 (61) | 7 (78) |
| | | AA | 4 (5) | 1 (3) | 9 (39) | 2 (22) |
| | *p* - value genotype | | 0.33 | ***0.009*** | ***0.01*** | ***0.01*** |
| | *p* - value allele | | 0.27 | ***0.02*** | 0.11 | ***0.056*** |

**Table 7**

Pre-surgical JPF scores by symmetry classification

| | n | Mean JPF Score | SD | *p* value* |
|---|---|---|---|---|
| Symmetric | 90 | 1.97 | 2.53 | <0.0001 |
| Asymmetric | 84 | 6.87 | 5.43 | |
| Group 1 | 9 | 3.75 | 4.09 | 0.009 |
| Group 2 | 35 | 6.94 | 5.46 | |
| Group 3 | 27 | 9.11 | 5.62 | |
| Group 4 | 13 | 4.00 | 3.61 | |

*Comparison between symmetric and asymmetric groups with unpaired *t*-test. Comparison across the four groups with ANOVA.

# Partie IV : Rôle des génotypes d'*ACTN3* dans l'équilibre de l'appareil manducateur

## I. Génotypes d'*ACTN3* et genèse des dysmorphoses dentofaciales

INTRODUCTION : Les alpha actinines sont des protéines d'ancrage des myofibrilles du muscle squelettique qui influencent les propriétés contractiles. *ACTN2* code pour l'α-actinine 2 qui est exprimé dans les fibres lentes de type I et rapides de type II alors que *ACTN3* code pour l'alpha actinine 3, exprimée uniquement dans les fibres rapides. Le polymorphisme nucléotidique *ACTN3 R577X* est présent chez environ 18% des Européens. Il en résulte une absence de la protéine alpha actinine 3, conduisant à une diminution l'activité contractile rapide, une amélioration des performances d'endurance et une réduction de la masse osseuse et la densité minérale osseuse. Nous avons étudié les génotypes d'*ACTN3* des polymorphismes nucléotidiques rs1815739 (*ACTN3 R577X*) et rs678397 et leur expression quantitative dans le muscle masséter chez des patients opérés d'une chirurgie orthognathique afin de déterminer leur association avec les caractéristiques architecturales maxillo-mandibulaires.

METHODE : Les caractéristiques biométriques, les échantillons de muscle masséter et de salive ont été obtenus pour 60 patients. Le génotypage des polymorphismes nucléotidiques d'*ACTN3* rs1815739 (*ACTN3 R577X*) et rs678397, la quantification de l'ARNm musculaire par RT-PCR et l'étude des propriétés histomorphométriques du muscle masséter ont été comparés pour déterminer les différences statistiques entre le génotype et le phénotype.

RESULTATS : Le niveau d'expression de l'ARNm musculaire était significativement différent pour les génotypes des polymorphismes d'*ACTN3* ($p<0.01$). La fréquence des génotypes d'*ACTN3* était significativement différente pour les dimensions sagittales et verticales de la malocclusion, l'association la plus claire étant l'augmentation de la fréquence du génotype *577XX* chez les patients présentant une dysmorphose dento-squelettique de classe II ($p<0.003$). Ce génotype était également associé à un diamètre significativement plus petit des fibres rapides de type II dans le muscle masséter ($p<0.002$).

CONCLUSION : *ACTN3 577XX* est surreprésenté chez les patients présentant une dysmorphose dento-squelettique de classe II, suggérant son influence au cours de la croissance osseuse au travers de modifications phénotypiques. *ACTN3 577XX* est sous-représenté chez les patients présentant une hypodivergence faciale, soulignant le rôle du phénotype musculaire masséterin sur la dimension verticale.

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# ACTN3 R577X Genotypes Associate with Class II and Deep Bite Malocclusions

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## Abstract

**Introduction—**α-actinins are myofibril anchor proteins which influence contractile properties of skeletal muscle. *ACTN2* is expressed in slow type I and fast type II fibers whereas *ACTN3* is expressed only in fast fibers. *ACTN3* homozygosity for the 577X stop codon (i.e. changing 577RR to 577XX - the R577X polymorphism) results in the absence of α-actinin-3 in about 18% of Europeans, diminished fast contractile ability, enhanced endurance performance and reduced bone mass or bone mineral density. We have examined *ACTN3* expression and genetic variation in masseter muscle of orthognathic surgery patients to determine genotype associations with malocclusion.

**Methods—**Clinical information, masseter muscle biopsies and saliva samples were obtained from 60 subjects. Genotyping for *ACTN3* SNPs, RT-PCR quantitation of muscle gene message and muscle morphometric fiber type properties were compared to determine statistical differences between genotype and phenotype.

**Results—**Muscle mRNA expression level was significantly different for *ACTN3* SNP genotypes ($p<0.01$). The frequency of ACTN3 genotypes was significantly different for sagittal and vertical classifications of malocclusion with the clearest association being elevated 577XX genotype in skeletal class II malocclusion ($p = 0.003$). This genotype also resulted in significantly smaller diameter of fast type II fibers in masseter muscle ($p = 0.002$).

**Conclusion—***ACTN3* 577XX is overrepresented in skeletal class II malocclusion, suggesting a biologic influence during bone growth. *ACTN3* 577XX is underrepresented in deep bite malocclusion, suggesting muscle differences contribute to variations in vertical facial dimensions.

Malocclusion often develops as a complex trait condition which is influenced by combinations of transcription and growth factors acting on bone, teeth and skeletal muscle.[1] Complex traits are quantitative or continuous conditions with a broad spectrum of presentations. For humans, variations in height, IQ, blood pressure and birth weight are complex quantitative traits which result from the interplay of genetic and environmental

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influences. One approach for identifying genes that contribute to the development of malocclusion is to consider those already known to influence musculoskeletal growth and function. Malocclusion is a complex musculoskeletal trait because masticatory muscle contributes to variations in the vertical dimension of facial growth.[2] Specifically, vertical facial dimensions are influenced by the size and proportion of muscle fiber types in masticatory muscles, with the majority of these studies being conducted by direct biopsy or indirect imaging studies of masseter muscle.[2-4] Genome-wide association analysis of skeletal muscle fiber types is underway,[5] and should add important information to the current Human Gene Map for Performance and Health-Related Fitness Phenotypes compendium, which summarizes gene variations that influence muscle size and strength.[6] Overall these gene association studies demonstrate that fiber type properties are influenced by genetic variation, which most commonly are single nucleotide polymorphisms (SNPs) in gene sequences which have functional consequences.[7] Unlike limb muscle, which is highly responsive to training and displays wide phenotypic variability with exercise and other environmental factors,[8] cranial muscles show less activity-related changes and are not typically subject to maximum force recruitments.[9-10]

Alpha-actinin-3 (*ACTN3*) is a particular gene of interest that influences muscle performance and fiber type proportions.[11] Alpha-actinins are cytoskeletal proteins which bind actin filaments in a variety of cell types. In skeletal muscle, α-actinin-2 and -3 crosslink actin filaments to dense bodies located in the Z-disk of the sarcomere, to help order the myofibril array during sarcomere contraction. Alpha-actinin-2 is found in all skeletal muscle fiber types, while Alpha-actinin-3 is restricted to most type II fast contracting fibers.[12] The genes that encode these two closely related isoforms are found on different chromosomes with *ACTN2* located on the long arm of chromosome 1, and *ACTN3* on chromosome 11.[13] A common nonsense mutation R577X identified in the *ACTN3* gene results in a lack of protein expression due to the production of a stop codon at residue 577.[14] About 18% of the European population is homozygous for the R577X change. Absence of α-actinin-3 is not associated with any obvious pathology, and since α-actinin-2 is still expressed in the fast fiber types, the functional role for the α-actinin-3 was first thought to be redundant.[15] Shortly thereafter, it became apparent that *ACTN3* genotype variations are important in human elite athletic performance. In a study comparing Australian Olympic athletes to controls, both male and female elite sprint athletes had higher frequencies of the 577R allele. Among females, elite sprint athletes also had higher 577RX heterozygote frequency and elite endurance athletes had lower 577RX frequency.[16] There was no comparable heterozygote genotype effect in male athletes. Subsequently, *ACTN3* allele and genotype frequencies have been investigated in at least ten other athletic and control populations.[17] These investigations support the conclusion that the 577RR genotype is more common in sprint/power athletes, but not that the X allele enhances endurance capability.[17] Overall, the literature indicates that the presence of α-actinin-3 enhances production of forceful, fast contraction in type II muscle fibers and that these genotypic effects may be influenced by gender.

Alpha-actinin-3 may also contribute to variations in muscle function by interaction with the signaling protein calcineurin to influence fiber type proportions during growth.[11] Alpha-

actinin-3 binds to calsarcin family signaling proteins located at the Z disc,[18] that in turn, bind to calcineurin to activate fiber type specific gene expression pathways which determine fiber types and size.[19] In a study of vastus lateralis muscle in young adult males, the RR genotype resulted in type IIX, fast contracting-fatigueable fibers, of larger size and greater number compared to the XX genotype.[11] Consequently, the men with RR genotype had significantly elevated leg muscle power. Type I, slow contracting fibers, also show variation with *ACTN3* genotype in vastus lateralis, with the percentage of type I fibers increasing in 577XX compared to RR genotype.[20] Therefore α-actinin-3 may act directly through structural functioning or cell-signaling pathways to alter composition and function of skeletal muscle.

Previous masseter muscle biopsy studies have demonstrated that increased size or proportion of type II fibers associates with skeletal deep bite malocclusion, and decreased type II fibers with skeletal open bite.[1-2] To further explore how genetic variation might influence masticatory muscle function and skeletal shape, this study sought to associate *ACTN3* genotypes with malocclusion classification and masseter muscle fiber type properties. To do so, we looked at two SNPs located at rs1815739 and rs678397. The first SNP, rs1815739 (R577X), is a Cytosine to Thymine transition at nucleotide 1,586 in exon 16 which converts an arginine to a stop codon at residue 577 and produces three genotypes CC (normal), TC (heterozygote) and TT (no α-actinin-3). The second SNP, rs678397, is a Cytosine to Thymine transition at nucleotide 15,193 in an *ACTN3* gene intron, which has no reported functional changes, and produces three genotypes CC (normal), TC and TT.

## MATERIAL AND METHODS

### Participants

Sixty subjects undergoing orthodontic and maxillofacial surgery treatment for correction of malocclusion were recruited from the University of Lille Department of Oral and Maxillofacial Surgery. Subjects were recruited after they had signed an informed consent, and the research protocol was validated by the French Independent Ethical Committee (named CPP), the Temple University and the University of Pittsburgh IRB Committees. Malocclusion classification was based upon the sagittal and vertical jaw repositioning required to execute the surgical treatment plan. Subjects were classified into one of six craniofacial morphologic groups that included one variation of sagittal skeletal jaw malocclusion, either Class II or III, and one variation of vertical skeletal jaw malocclusion, open, deep or normal - bite relationship. Thirty one non-treated Class I control subjects without malocclusion came from the Dental Registry and DNA Repository at the University of Pittsburgh which included only subjects of European descent, to be comparable to the French subjects with malocclusion. Saliva samples from all subjects were stored in Oragene® kits and used for DNA extraction and posterior genotyping. For the malocclusion subjects, muscle samples were also collected from the deep anterior area of masseter muscle as a bilateral sagittal split osteotomy of the mandible was performed. After snap freezing, muscle samples were stored at -80$^0$C prior to histologic and gene expression analysis.[1-2]

### Genotyping

Two SNPs in *ACTN3* were selected for genotyping on all subjects, rs1815739 (the R577X SNP) and rs678397 (a SNP not know to have functional consequences) and tested to determine if specific allelic variants are over-represented in subjects with malocclusion sub-classifications using TaqMan chemistry and end-point analysis in an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems, Foster City, CA), as described previously.[21] Thirty-three additional anonyms SNPs were genotyped to assess the presence of population substructure among the controls selected.

### Fiber type histomorphometric analysis of masseter muscle

Frozen muscle was cryosectioned at 10 $\mu$m thickness to obtain serial cross sectional slices, and sections were mounted on glass microscope slides for immunostaining with five antibodies specific for myosin heavy chain (MyHC) isoforms; anti-Type I, anti-Type II, anti-Type IIA, anti-Type neonatal and anti-α-cardiac (atrial) as described previously.[22-23] We classified masseter fibers into 4 fiber type groups as type I, type hybrid (containing both type I and II MyHCs), type II containing only type IIA and/or IIX MyHCs and type neonatal - atrial that contained the neonatal and/or α–cardiac MyHCs in combination with other type I and II isoforms. Type I fibers are slow contracting and fatigue resistant, used most commonly to maintain postural freeway space. Type II fibers are fast contracting and are either fatigue resistant (type IIA) or fatigueable (type IIX). The hybrid fibers are a very unusual and distinctive fiber type found in masseter muscle, which combines slow and fast contractile properties.[23] Hybrid fibers are more commonly found in certain states of skeletal muscle pathology, although they are sometimes present in normal healthy limb muscle. In human masticatory muscles however, hybrid fibers are always present as a common fiber type. In addition, α-actinin-3 was identified across fiber types by staining with a rabbit IgG monoclonal antibody (EP2531Y), commercially available from Origene Technologies, Inc. For fiber type classification, only tissue section series with consistent antibody reactions for all stains and acceptable morphology of muscle fibers, which were clearly in transverse section, were used. All fibers within the selected areas were type - classified and their cross - sectional areas measured with Image J image - analysis software available from the National Institutes of Health. Tests for measurement error included intra-rater reliability in determination of fiber area (by repeating morphometric tracing of all fiber areas in one biopsy by one examiner), which resulted in an $R^2$ value of 0.94. We compared differences in fiber type properties between all of the malocclusion subjects that were genotyped as either 577XX or RR.

### Quantification of Actinin mRNA

RNA was isolated from the remaining muscle after cryosectioning with TRIzol™ reagent and quantified by absorbance at $A_{260}$ as described previously.[24] *ACTN2* and *ACTN3* were quantified by TaqMan® quantitative real time PCR (qRT-PCR). Reactions, in triplicate, contained RNA from masseter muscle or an adult skeletal muscle reference standard, TaqMan RNA-to-$C_T$ 1-Step reagent and an Applied Biosystems expression assay set for either *ACTN2* (#Hs01100111_g1), *ACTN3* (#Hs00153809_m1) or internal control HPRT1 (#Hs02800695_m1) for normalization. RNA was expressed as relative quantities determined

by the comparative $C_T$ ($\blacktriangle\blacktriangle C_T$) method that measures fold - difference between normalized quantities of target in the sample and in the reference standard. A limited number of subjects were included in the mRNA quantification to determine if there were differences in message expression between *ACTN3* genotypes rs1815739 and rs678397 for 13 of the study subjects.

### Statistical Analysis

Chi-square or Fisher's exact tests were used to assess Hardy-Weinberg equilibrium and compare allele and genotype distributions between individuals with malocclusion and non-treated Class I control subjects. Student's *t* tests were used to compare differences for fiber type mean fiber area, fiber number and percent occupancy measurements between *ACTN3* genotypes. An ANOVA was used to compare differences in malocclusion classifications for *ACTN3* genotypes between malocclusion and control subjects. A separate ANOVA was conducted to determine if *ACTN2* and *3* mRNA expression levels were different by R577X genotype.

## RESULTS

### ACTN3 Genotype

We recruited sixty orthognathic surgery patients who were systemically healthy and without genetic craniofacial syndromes, other growth disturbances or reported trauma. They were 42 females and 18 males with the average age of 23.5 ± 9.9 years, including 18 Class II open bite, 7 Class III open bite; 13 Class II deep bite, 6 Class III deep bite; 12 Class II normal bite and 4 Class III normal bite malocclusion. R577X genotype frequencies for the entire malocclusion group was 14% CC, 63% TC and 23% TT, with the reported European population frequency of 18% for TT.[16] Lateral cephalograms of one Class III open bite subject with R577X CC genotype (A) and one Class II open bite subject with TT genotype (B) are shown in **Figure 1**. Because we hypothesized that the *ACTN3* SNPs would differ between sagittal and vertical malocclusion classification we compared SNP frequency for Class II vs Class III to Class I controls (**Table I**) and secondly between open, normal and deep bite (**Table II**). Overall, only a small number of genotypes were undetermined, ranging from 0 to 3 for each comparison.

No significant allele frequency differences were found between cases and controls in the 31 anonymous SNPs genotyped (data not shown), and we proceeded to test differences in *ACTN3* genotypes between cases and controls. There were no deviations from the Hardy-Weinberg equilibrium in the samples and no statistically significant differences between cases and controls in regards to age and sex. Chi-square tests revealed very significant differences between Class II malocclusion vs. controls for genotypes and alleles at rs1815739 and rs678397 (**Table I**). There were no significant differences for Class III malocclusion. In vertical dimension comparisons, there were very significant differences for genotypes and alleles at rs678397 (**Table II**). For rs1815739, the TT and CC genotypes were underrepresented in subjects with deep bite malocclusion.

### RNA Expression Levels

Although the 577XX should result in no α-actinin-3, we assayed mRNA levels for *ACTN2* and *ACTN3* to determine if gene expression levels differed for this SNP. In doing so, we wanted to test whether *ACTN2* expression is increased when α-actinin-3 was absent. Relative RNA was quantified in masseter muscle from 13 malocclusion subjects with differing R577X genotypes (**Table III**). A three way ANOVA between genotypes found no difference in gene expression for *ACTN2* ($p = 0.84$) and significant differences in gene expression for *ACTN3* ($p = 0.003$). Based on this result it is expected that the R577X CC genotype should have a full complement of no α-actinin-3, the TC genotype an intermediate amount and TT no detectible no α-actinin-3, which supports previous findings.[14] *ACTN2* expression remained relatively consistent regardless of changes in *ACTN3* genotype.

### Fiber Type Differences Associated with ACTN3 R577X Genotype

We compared differences in fiber type properties from masseter muscle between 12 subjects genotyped at rs1815739 as TT and 8 subjects genotyped as CC to determined how normal or absent alpha-actininn-3 protein expression affected muscle phenotype. Since we know from previous studies that the size and occupancy of type II fibers influence the length of vertical anterior facial dimension, it was important to know what malocclusion types are being compared along with *ACTN3* genotypes. The TT subjects included 10 Class II, 3 Class III, 6 open bite, 5 normal bite and 2 deep bite malocclusions and the CC subjects included 4 Class II, 4 Class III, 5 open bite, 1 normal bite and 2 deep bite malocclusions. So for vertical dimension classification in both groups, open bite malocclusion was the most common feature, and was larger in proportion for the CC group. The mean type II fiber area and muscle percent occupancy composition was larger in subjects with CC genotype (**Table IV, Figure 2**). The mean type II fiber area was significantly smaller for the TT genotype, being on average less than half the size of type II fibers in the CC genotype ($p = 0.002$). (**Table IV, Figure 3**). There were however larger numbers of type II fibers in the TT genotype, which did not produce a significant difference in type II fiber numbers between groups. Although type II fibers had larger occupancy in the CC group, the differences were not significant. There was an almost significant difference between type I fiber occupancy, with the TT group having a higher percent occupancy for type I fibers. For CC genotype, fiber staining profiles demonstrate that no α-actinin-3 is expressed in all fibers that contain IIA or IIX MyHCs. This is demonstrated by the similar staining pattern and intensity for fast MyHC antibody (**Figure 2A**) and alpha-actinin-3 antibody (**Figure 2D**). For TT genotype, there was no detectable alpha-actinin-3 in masseter muscle (**Figure 3D**). There was also a lack of neonatal and/or alpha–cardiac MyHC proteins in masseter muscle from subjects with TT genotype. Of the 8 subjects studied with CC genotype, three had limited expression of alpha–cardiac MyHC and four had limited expression of neonatal MyHC.

## DISCUSSION

The *ACTN3* 577XX genotype is a common nonsense mutation which has been studied throughout world populations, usually comparing elite athletes to the general population.[17,20] There is consensus that the presence of alpha-actinin-3 protein is advantageous in elite athletic performance for sprinting and power lifting, and there is

limited evidence that lack of no α-actinin-3 protein enhances human endurance activities.[17] In mouse models however, there is clear evidence that α-actinin-3 co-localizes with glycogen phosphorylase and enhances oxidation of carbohydrates during exercise. In knockout mice, type II muscle fibers have reduced free glucose and shift from glycolytic metabolism towards a more oxidative metabolism similar to type I fibers.[25] Since the human studies conducted so far have been on appendicular muscle function, one cannot rule out that there are significant shifts in cranial muscle energy metabolism between *ACTN3* genotypes.

We were able to compare α-actinin-3 and mRNA expression levels in masseter muscle to *ACTN3* R577X genotypes in 60 subjects to determine if differences in protein function might contribute to development of malocclusion. Although we hypothesized that the main effect of α-actinin-3 loss (TT) would be smaller type II fibers and open bite malocclusion, the most distinctive finding was that TT was overrepresented in Class II skeletal malocclusion ($p = 0.003$). There were too few Class III subjects, however, to determine genotypic effects for this malocclusion (**Table I**). It has recently been discovered that alpha-actinin- is expressed in bone osteoblasts. Both knockout mice and postmenopausal women with TT genotype have significantly decreased bone mineral density.[26] Unlike muscle, α-actinin-2 is not expressed in osteoblasts and therefore cannot become a functional substitute when α-actinin-3 is absent. Although the molecular role that α-actinin-3 might play in bone mineralization is at present unknown, osteoblasts cultured from *ACTN3* knockouts have abnormal gene expression with *Enpp1* (ectonucleotide pyrophosphotase/phosphodiesterase) increased by 1.45 fold. *Enpp1* is an important negative regulator of bone mineralization, and cultured osteoblasts with elevated *Enpp1* expression have reduced mineral formation.[27] It is possible that differences in bone growth that occur with TT genotype contribute to Class II skeletal malocclusions when *Enpp1* expression is elevated. A second possibility is that SNPs in *Enpp1* play an additional role, since *Enpp1* polymorphisms are associated with differences in height, hip geometric indices and facial morphology.[28-30]

As expected, loss of α-actinin-3 with R577X TT genotype did result in significantly smaller type II fiber diameter in masseter muscle ($p = 0.002$). Type II fiber number was increased for TT compared to CC, but these differences were insignificant (**Table IV**). Mouse knockout experiments have also demonstrated reduced diameter for type II fibers, but without changes in fiber numbers.[25] The TT genotype was underrepresented in deep bite subjects vs. controls ($p = 0.02$) (**Table II**). This most likely resulted in increased type II fiber percent occupancy in the deep bite group compared to the open and normal bite groups and provides a mechanical explanation for how the genotypes affect vertical facial dimension.

The *ACTN3* SNP at rs678397 is also associated with significant differences in sagittal and vertical malocclusion classifications (**Table I and II**). Genotypes and alleles at rs678397 are significantly different for Class II malocclusion (**Table II**) and for all vertical dimension bite malocclusions. The SNP is located in an intron area not known to change protein levels or functioning. The possibility remains that this intronic SNP alters protein levels. The initial hypothesis is that rs678397 is in linkage disequilibrium with another undermined *ACTN3* variant that impacts the function of α-actinin-3.

Experimental subjects were from Lille, France and control subjects were European Americans without malocclusion from Pittsburgh, Pennsylvania. *ACTN3* allele frequency varies with global latitudinal gradient, with higher frequency of 577XX occurring at increased latitude.[31] Despite no evidence that there is undetected population substructure in the study, our comparison may be somewhat biased in that Lille is geographically $10^0$ latitude higher than Pittsburgh. Further studies which include Class I control subjects from Lille will be necessary to confirm that *ACTN3* genotypes contribute to development of malocclusion. Of course SNPs in many other genes are influencing the diversity of muscle and jaw properties and ultimately must be considered together to understand musculoskeletal heritability. *Enpp1* may be a key genetic influence and needs further careful consideration. In addition to the bone effects described above, it also binds directly to skeletal muscle insulin receptors, and a polymorphism is known to produce insulin resistance.[32-33] Over time, insulin resistance alters the proportion of type I versus type II skeletal muscle fibers,[34] which could also influence the vertical dimension of malocclusion.[2-4] How *ACTN3* and *ENPP1* gene expression levels interact in maintenance of muscle and bone is yet to be investigated, but could have insightful findings for conditions such as osteoporosis, obesity, diabetes and malocclusion.[35] In the future, orthodontists may use genetic findings derived from saliva tests of individual patients for a personalized medicine approach to treatment planning. If SNPs like the *ACTN3 R*577X polymorphism are detected as part of routine diagnosis and treatment planning, a more personalized and specific treatment plan might be developed to produce a more favorable treatment result.

## CONCLUSIONS

1. Alpha-actinin-3 enhances forceful, fast skeletal muscle contraction and strength. A common ACTN3 polymorphism, R577X, results in alpha-actinin-3 protein absence, changes in fiber type proportions, muscle metabolism and bone mineralization. This study demonstrated that R577X associates with Class II and deep bite skeletal malocclusions.
2. Loss of alpha-actinin-3 was accompanied by significantly smaller type II fiber diameter in masseter muscle. Although we expected this decrease in diameter to result in an association between 577XX and open bite malocclusion, significant genotype associations were demonstrated with deep bite malocclusion versus controls. This is most likely resulted from increased type II fiber percent occupancy in masseter muscle, even though average type II fiber diameter was smaller.
3. RT-PCR experiments demonstrated that as *ACTN3* mRNA expression decreased to almost undetectable with 577XX genotype, *ACTN2* expression levels remained unchanged, suggesting that in masseter muscle alpha-actinin-2 may not compensate for loss of alpha-actinin-3.
4. Two SNPs in ACTN3 at rs1815739 and rs678397 had statistically significant differences between Class II malocclusion and controls. These SNPs had no significant association with Class III malocclusion, which may be the result of limited numbers of Class III subjects in the study. These findings suggest that bone

growth may be altered with *ACTN3* genotype for Class II subjects in our population.

5. Another study has demonstrated that *ACTN3* knock-out mice have significant decreases in bone mineral density. Further, absence of alpha-actinin-3 in bone resulted in increased expression of Enpp1, a negative regulator of mineralization. These relationships may be important to the development of Class II malocclusions in humans and merit further investigation.

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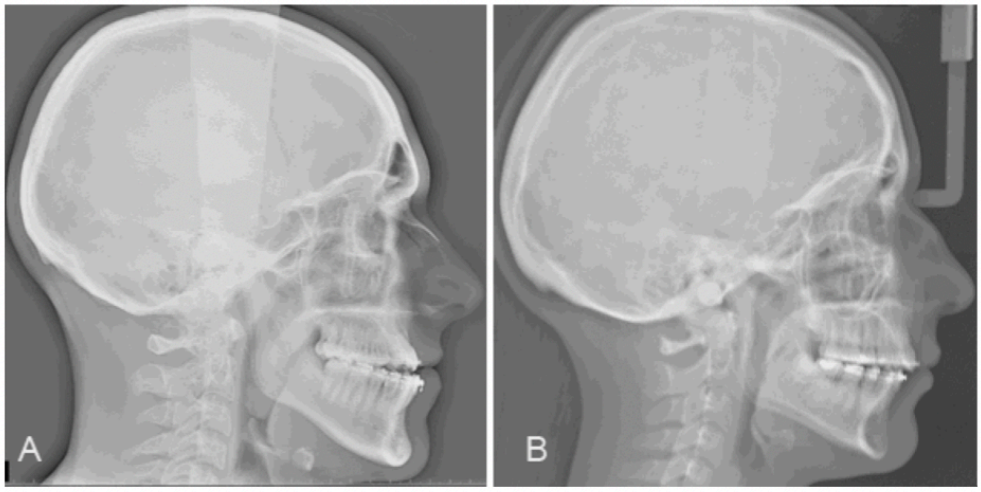


**Figure 1.**
Lateral cephalograms of two *ACTN3* - rs1815739 genotyped subjects. Left subject (A) is a Class III open bite malocclusion with CC genotype and right subject (B) is a Class II open bite with TT genotype.

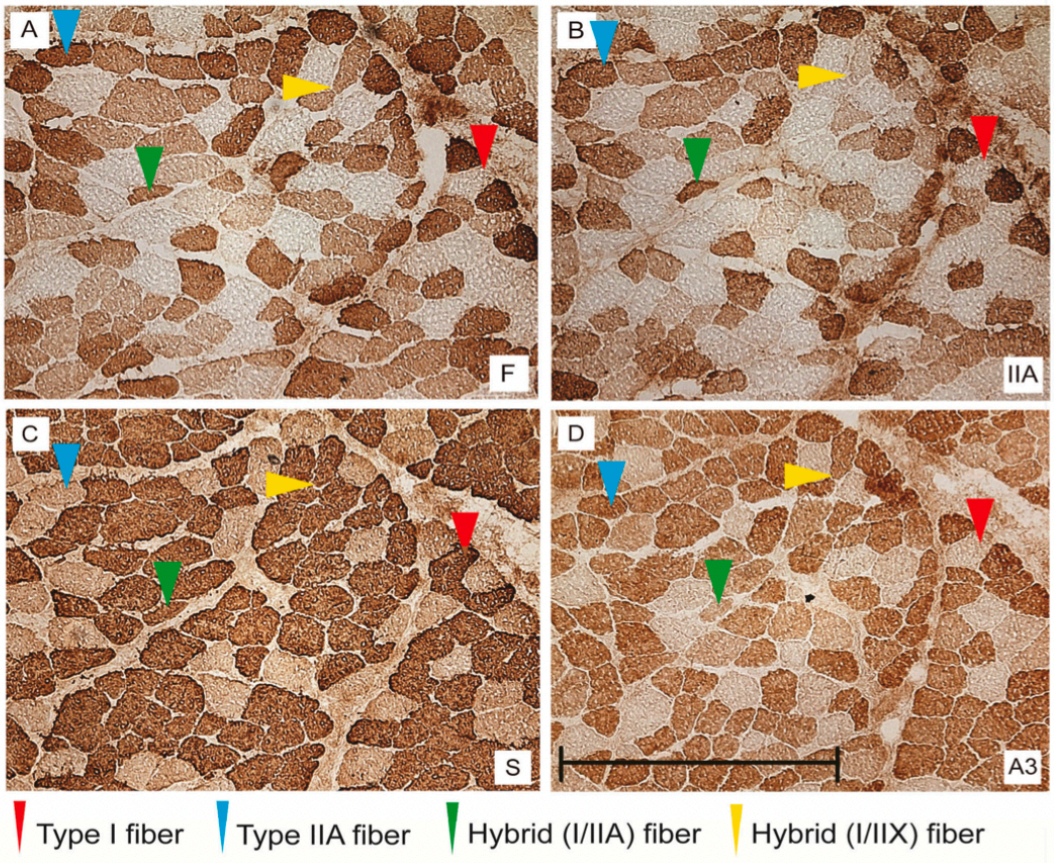


**Figure 2.**

Serial sections of masseter muscle from an *ACTN3* – rs1815739 CC genotyped subject stained for Myosin Heavy Chain (MyHC) and alpha-actinin-3 protein antibodies. A. anti-fast MyHC antibody stain, B. anti-IIA, C. anti-type I and D. anti – alpha-actinin-3. Arrows mark fibers by type classification. Alpha-actinin-3 protein is present in all type II and hybrid fibers, but not in type I fibers. Bar = 1000$\mu$m.

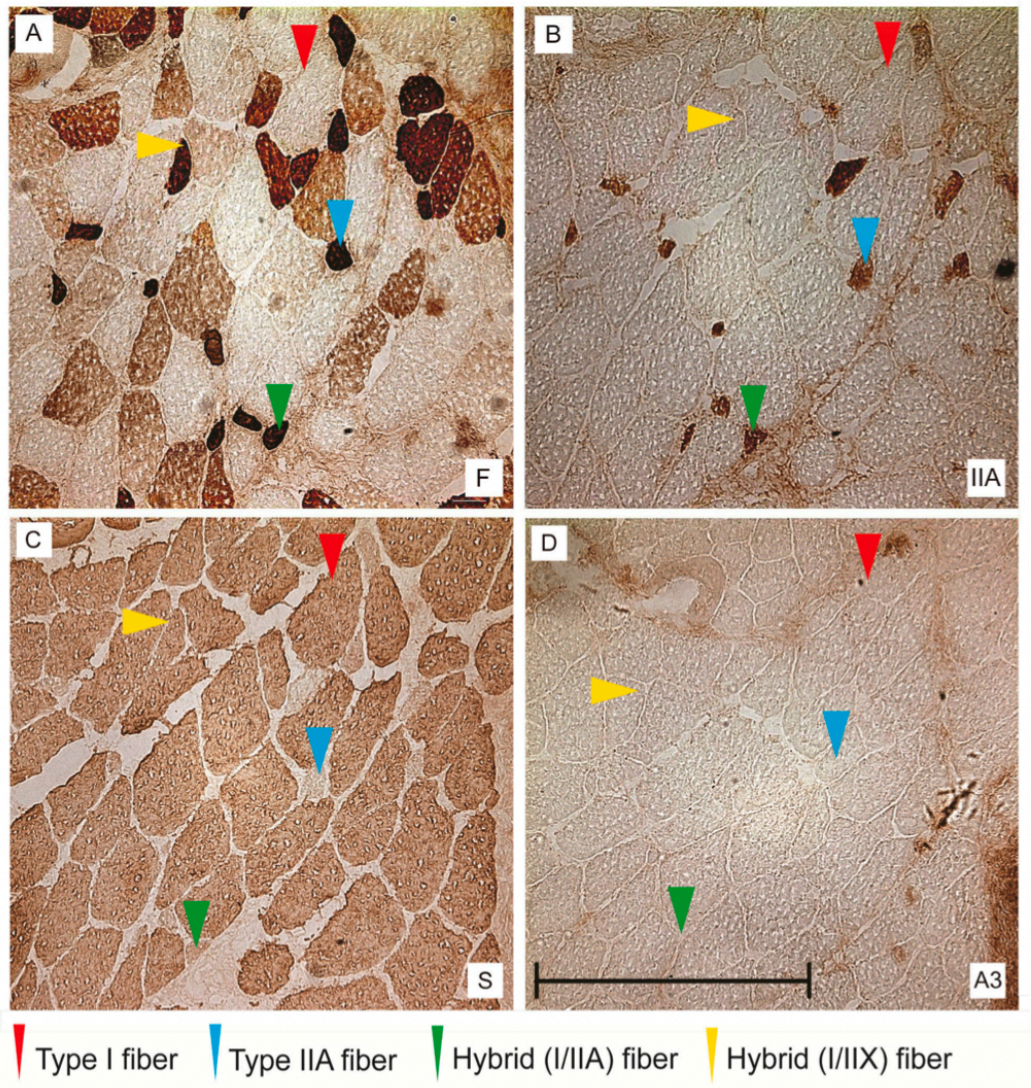


**Figure 3.**

Serial sections of masseter muscle from an *ACTN3* – rs1815739 TT genotyped subject stained for MyHC and alpha-actinin-3 antibodies. A. anti-fast MyHC antibody stain, B anti-IIA, C. anti-type I and D. anti – alpha-actinin-3. Arrows mark fibers by type classification. There is no alpha-actinin-3 antibody staining of hybrid or type II fibers. The type IIA fibers have relatively small fiber diameter, compared to other fiber types, and to type IIA fibers stained for the CC genotype subject (Fig. 2B). Bar = 1000$\mu$m.

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## Table I

*ACTN3* Genotype % by Malocclusion Class Compared to Normal Controls

| | | *Genotypes* | | | | *Total n* | | p *value* | |
|---|---|---|---|---|---|---|---|---|---|
| ***SNP Marker*** | **Phenotype** | **CC** | **TC** | **TT** | **undetermined** | **n** | | **Genotypes** | **Alleles** |
| ***ACTN3* rs1815739** | Class III | 4 (25%) | 9 (56%) | 3 (19%) | 1 | 17 | | 0.39 | 0.28 |
| | Class II | 4 (10%) | 27 (66%) | 10 (24%) | 2 | 43 | 60 | 0.003** | 0.009** |
| **Control** | Class I | 14 (45%) | 12 (39%) | 5 (16%) | | 31 | | | |

| | | **CC** | **TC** | **TT** | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***ACTN3* rs678397** | Class III | 4 | 9 | 3 | 1 | 17 | | 0.11 | 0.04 |
| | Class II | 4 (10%) | 24 (60%) | 12 (30%) | 3 | 43 | 60 | 0.003** | 0.000005** |
| **Control** | Class I | 17 (55%) | 12 (39%) | 2 (6%) | | 31 | | | |

** $p < 0.01$

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## Table II

*ACTN3* Genotype % by Vertical Dimension Classification

| *Marker (Gene)* | Phenotype | *Genotypes* | | | | | Total n | *p-value* | |
|---|---|---|---|---|---|---|---|---|---|
| | | CC | TC | TT | undetermined | N | | genotypes | alleles |
| ***ACTN3* rs1815739** | Normal bite | 1 (7%) | 10 (62%) | 5 (31%) | 0 | 16 | | 0.6 | 0.4 |
| | Open bite | 5 (22%) | 12 (52%) | 6 (26%) | 2 | 25 | | 0.2 | 0.41 |
| | Deep bite | 2 (11%) | 14 (78%) | 2 (11%) | 1 | 19 | 60 | 0.02* | 0.16 |
| **Control** | Normal occlusion | 14 (45%) | 12 (39%) | 5 (16%) | | 31 | | | |
| | | **CC** | **TC** | **TT** | **undetermined** | | | | |
| ***ACTN3* rs678397** | Normal bite | 1 (6%) | 9 (56%) | 6 (38%) | 0 | 16 | | 0.001** | 0.0002** |
| | Open bite | 5 (21%) | 13 (54%) | 6 (25%) | 1 | 25 | | 0.02* | 0.005** |
| | Deep bite | 2 (12%) | 11 (69%) | 3 (19%) | 3 | 19 | 60 | 0.02* | 0.009** |
| **Control** | Normal occlusion | 17 (55%) | 12 (39%) | 2 (6%) | | 31 | | | |

*$p < 0.05$
**$p < 0.01$

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## Table III

Differences in gene expression for *ACTN2* and *3* by R577X Genotype

| *Gene* | *Mean mRNA expression (average RQ)* | | | *p-value* |
|---|---|---|---|---|
| | **(rs1815739) CC** | **TC** | **TT** | |
| ***ACTN2*** | 0.514 (n=4) | 0.635 (n=5) | 0.55 (n=4) | 0.84 |
| ***ACTN3*** | 0.957 (n=4) | 0.387 (n=5) | 0.02 (n=4) | 0.003** |

** $p < 0.01$

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## Table IV

Fiber Type Differences between *ACTN3* R577X Genotype (TT vs. CC)

| *Fiber Type* | *Mean Fiber Area ($\mu m^2$) ± CI* | | | *Fiber Number* | | | *Fiber Occupancy (%)* | | |
|---|---|---|---|---|---|---|---|---|---|
| | **TT** | **CC** | ***p* value** | **TT** | **CC** | ***p* value** | **TT** | **CC** | ***p* value** |
| **Type I** | 1575.8 ± 646 | 2360.9 ± 60.7 | 0.11 | 41.4 ± 26.9 | 70.5 ± 42.5 | 0.25 | 49.7 ± 10.8 | 48.09 ± 8.6 | 0.08 |
| **Hybrid** | 1313.8 ± 63.1 | 1277.3 ± 77.2 | 0.89 | 22.4 ± 10.3 | 31.9 ± 24.2 | 0.41 | 31.4 ± 10.3 | 19.42 ± 6.9 | 0.452 |
| **Type II** | 634.9 ± 244.8 | 1421.6 ± 06.3 | **0.002**** | 58.2 ± 41.4 | 31.7 ± 13.6 | 0.344 | 17.1 ± 9.9 | 22.4 ± 7.75 | 0.439 |

** $p < 0.01$

## II. Association entre génotypes d'*ACTN3* et gènes OPPERA liés à la douleur

INTRODUCTION : Nous avons étudié une population de patients bénéficiant d'une chirurgie orthognathique afin de déterminer quel était le rôle de la variation de l'expression génique et du génotype du muscle masséter dans le développement d'une dysmorphose dentofaciale et d'une dysfonction temporomandibulaire. *ACTN3* est un gène d'intérêt dont le polymorphisme *ACTN3 R577X* entraîne une perte de protéine alpha actinine 3, une réduction de l'intégrité structurelle du disque Z de la myofibre dans le muscle squelettique et une diminution de l'activité des ostéoblastes/ostéoclastes dans la formation osseuse. Compte tenu de la prévalence assez élevée des DTM dans cette population (30%), nous avons étudié l'expression des gènes liés aux processus douloureux, précédemment identifiés dans l'étude *« Orofacial Pain: Prospective Evaluation and Risk Assessment Study » (OPPERA)*.

METHODE : Après avoir recueilli des échantillons de muscle masséter et d'ADN de salive de patients au cours d'une chirurgie orthognathique, nous avons identifié des associations entre le génotype, l'expression des gènes musculaires, les propriétés du type de fibre, la classification des malocclusions, l'asymétrie faciale, le sexe et les dysfonctions temporomandibulaires. Le génotype et les niveaux d'expression génique ont été déterminés par la technique de PCR quantitative en temps réel (TaqMan®). L'histomorphométrie des fibres musculaires a été réalisée sur des coupes transversales de tissus colorés avec des anticorps spécifiques à la chaîne lourde de la myosine. Un questionnaire sur la douleur et la fonction manducatrice et un examen clinique spécifique ont été utilisés pour caractériser les dysfonctions temporomandibulaires. La dysmorphose dentofaciale a été classifiée à l'aide d'un bilan céphalométrique.
Dans une analyse pilote séparée, des échantillons musculaires ont été analysés pour les différences d'expression génique sur des puces d'expression de *microarray Affymetrix HT2.0* contenant 70 534 transcrits. L'analyse en composantes principales et les corrections du taux de fausses découvertes ont été appliquées aux comparaisons avec le logiciel *Partek Genomics Suite*®.

RESULATS : Nous avons identifié des associations entre le génotype d'*ACTN3* et les dysmorphoses dento-squelettiques de classe II (p=0.003) et de caractère *deep-bite* (p=0.03), le

type de fibre musculaire du muscle masséter (p=0.02) et une tendance à la significativité pour la présence d'une dysfonction temporomandibulaire, qui était souvent limitée à des myalgies (p=0.08). L'analyse globale de l'expression génique a identifié des différences significatives pour environ 200 gènes *OPPERA* liés aux processus douloureux chez les patients présentant une dysmorphose dentofaciale avec une asymétrie faciale et une dysfonction temporomandibulaire, par rapport aux sujets présentant uniquement une dysmorphose dentofaciale. L'expression différentielle dans le muscle masséter pour l'un de ces gènes, *CACNA2D1* (sous-unité alpha-2 / delta-1 du canal calcique voltage-dépendant, actif dans la douleur neuropathique), a été confirmée par RT-PCR quantitatives, par sexe (p=0.0008) et entre les femmes avec et sans myalgie (p=0.05).

CONCLUSION : Ces résultats indiquent que le génotype d'*ACTN3* contribue de manière significative au développement d'une dysmorphose dentofaciale en tant qu'affection musculo-squelettique. Les patients présentant une dysmorphose dentofaciale, en particulier les femmes, ont une prévalence élevée de dysfonctions temporomandibulaires, diagnostiquées cliniquement, principalement à type de myalgie. Des différences dans les taux de protéine alpha actinine 3 pourraient prédisposer le muscle à des dommages induits par la contraction ou à une nociception altérée par la calcineurine.

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MOLECULAR PAIN

POSTER PRESENTATION Open Access

# Associations between *ACTN3* and OPPERA pain-related genes in malocclusion

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*From* Seventh Scientific Meeting of The TMJ Association, Genetic, Epigenetic, and Mechanistic Studies of Temporomandibular Disorders and Overlapping Pain Conditions
Bethesda, MD, USA. 7-9 September 2014

**Background**

We have investigated an orthognathic surgery population to determine how variation in masticatory muscle gene expression and genotype plays a key role in development of both jaw-deformation malocclusion and temporomandibular joint disorders (TMD). A gene of particular interest is *ACTN3* since the common R577X polymorphism results in α-actinin-3 protein loss, reduced myofiber Z-disc structural integrity in skeletal muscle and decreased osteoblast/osteoclast activity in bone formation. Secondly, since the prevalence of TMD in this population is quite high (30%) we sought to determine if genes related to pain processes—previously identified in the Orofacial Pain: Prospective Evaluation and Risk Assessment Study (OPPERA) were differentially expressed.

**Methods**

After obtaining masseter muscle and saliva-DNA samples from subjects during orthognathic surgery, we identified associations between genotype, muscle gene expression, fiber type properties, malocclusion classification, facial asymmetry, gender and TMD. Genotype and gene message quantities were determined using TaqMan chemistry. Morphometry of muscle fiber types was conducted on tissue cross sections stained with myosin heavy chain–specific antibodies using NIH Image software. Jaw Pain and Function questionnaire and clinical examinations were used to diagnose TMD. Malocclusion diagnosis was determined by the type of treatment plans executed during surgery.

In a separate pilot analysis muscle samples were analyzed for gene expression differences on Affymetrix HT2.0 microarray expression chips containing 70, 534 transcripts. Principal Components Analysis and False Discovery Rate corrections were applied to comparisons with Partek Genomics Suite software.

**Results**

We identified associations between *ACTN3* genotypes and skeletal class II ($p=0.003$) and deep bite ($p=0.03$) malocclusions, masseter muscle fiber type properties ($p=0.02$) and an almost significant association for presence of TMD, which was often limited to masticatory muscle pain ($p=0.08$). Global gene expression analysis identified significant differences for approximately 200 OPPERA pain process genes in subjects with asymmetry and TMD, compared to subjects with malocclusion only. Differential expression in masseter muscle for one of these genes, *CACNA2D1* (voltage-dependent calcium channel subunit alpha-2/delta-1, active in neuropathic pain), was confirmed with additional quantitative RT-PCR experiments by gender ($p=0.0008$) and between women with and without myalgia ($p=0.05$).

**Conclusions**

These results indicate that *ACTN3* genotypes make significant contributions in the development of malocclusion as a musculoskeletal condition. Dentofacial deformity subjects, especially females, have a high prevalence for TMD, diagnosed clinically as masticatory muscle myalgia. Differences in α-actinin-3 protein levels may predispose muscle to contraction-induced damage, or altered calcineurin-mediated nociception.

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## III. Remodelage condylien, asymétrie craniofaciale et génotypes d'*ACTN3*

INTRODUCTION : L'asymétrie craniofaciale, le remodelage du condyle mandibulaire et les dysfonctions temporomandibulaires sont des comorbidités fréquentes des dysmorphoses dentofaciales, mais leur étiologie reste mal comprise. Nous avons évalué l'asymétrie, la morphologie condylienne et la santé articulaire temporomandibulaire, et étudié les polymorphismes de gènes d'intérêts dans une cohorte de 128 patients ayant bénéficié d'une chirurgie orthognathique pour corriger une dysmorphose dentofaciale.

METHODE : Les analyses radiographiques céphalométriques pré-chirurgicales, l'évaluation clinique de la douleur et des troubles de la fonction articulaire par des échelles standardisées, et l'analyse du génotype de polymorphismes nucléotidiques d'*ACTN3* et *ENPP1* à partir d'échantillons salivaires ont été comparés pour évaluer leurs interrelations. *ACTN3* code pour l'alpha actinine 3, une protéine d'ancrage des myofibrilles du muscle squelettique qui influencent les propriétés contractiles. *ENPP1* code pour l'ectonucleotide pyrophosphatase / phosphodiesterase de type 1, une ecto-enzyme transmembranaire hydrolysant des molécules extracellulaires ayant des liaisons pyrophosphate ou phosphodiester. Dans les chondrocytes et les ostéoblastes, cette protéine régule la minéralisation osseuse.

RESULTATS : Près de la moitié des patients présentaient une asymétrie nécessitant une correction chirurgicale, qui pouvaient être subdivisée en quatre modèles morphologiques distincts. Une asymétrie de modelage condylien était significativement plus élevée dans le modèle d'asymétrie craniofaciale, mais avait le plus souvent un motif imprévu. Souvent, des condyles plus longs ou plus gros se trouvaient du côté du *ramus* mandibulaire le plus court. Les sujets avec un *ramus* plus long mais des condyles dimensionnellement plus petits étaient plus susceptibles d'avoir des symptômes de dysfonction temporomandibulaire auto-déclarés ($p=0.023$) et un diagnostic clinique positif ($p=0.0000001$), principalement à type de myalgie. Nous avons retrouvé une association très significative ($p<0.0001$) entre les allèles *R577X* et le remodelage condylien dans le groupe d'asymétrie faciale typique, l'allèle X (polymorphisme nul) étant plus élevé lorsque le modelage du condyle est normal. Il y avait d'autres associations significatives pour les génotypes d'*ACTN3* rs1671064 et rs678397.

CONCLUSION : L'asymétrie squelettique, un remodelage anormal du condyle mandibulaire et les dysfonctions temporomandibulaires sont des comorbidités communes et interdépendantes de nombreuses dysmorphoses dentofaciales. Des adaptations fonctionnelles musculo-squelettiques déséquilibrées et des influences génétiques ou épigénétiques contribuent à leur étiologie et nécessitent des études plus approfondies.

PLOS ONE

RESEARCH ARTICLE

# Condyle modeling stability, craniofacial asymmetry and ACTN3 genotypes: Contribution to TMD prevalence in a cohort of dentofacial deformities

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## Abstract

Craniofacial asymmetry, mandibular condylar modeling and temporomandibular joint disorders are common comorbidities of skeletally disproportionate malocclusions, but etiology of occurrence together is poorly understood. We compared asymmetry, condyle modeling stability and temporomandibular health in a cohort of 128 patients having orthodontics and orthognathic surgery to correct dentofacial deformity malocclusions. We also compared *ACTN3* and *ENPP1* genotypes for association to clinical conditions. Pre-surgical posterior-anterior cephalometric and panometric radiographic analyses; jaw pain and function questionnaire and clinical examination of TMD; and SNP-genotype analysis from saliva samples were compared to assess interrelationships. Almost half had asymmetries in need of surgical correction, which could be subdivided into four distinct morphological patterns. Asymmetric condyle modeling between sides was significantly greater in craniofacial asymmetry, but most commonly had an unanticipated pattern. Often, longer or larger condyles occurred on the shorter mandibular ramus side. Subjects with longer ramus but dimensionally smaller condyles were more likely to have self-reported TMD symptoms ($p = 0.023$) and significantly greater clinical diagnosis of TMD ($p = 0$ .000001), with masticatory myalgia most prominent. Genotyping found two significant genotype associations for *ACTN3* rs1671064 (Q523R missense) $p = 0.02$; rs678397 (intronic SNP) $p = 0.04$ and one significant allele association rs1815739 (R577X nonsense) $p = 0.00$. Skeletal asymmetry, unusual condyle modeling and TMD are common and interrelated components of many dentofacial deformities. Imbalanced musculoskeletal functional adaptations and genetic or epigenetic influences contribute to the etiology, and require further investigation.

## 1. Introduction

Growth and stability of the mandibular condyle is essential for attainment and maintenance of mandibular size and morphology. Agenesis, trauma, local infectious pathologies and juvenile idiopathic arthritis all produce similar and distinctive mandibular morphologic disruptions, due to decreased growth in length and normal attainment of transverse width. [1] These condylar growth deficiencies result in skeletal class II open bite malocclusions characterized by a downward and backward growth rotation at the joint articulation and a pronounced antigonial notch. Variability in diminished mandibular length and severity of the dysmorphology is directly related to the chronologic age at which condylar disturbance is first encountered, as demonstrated in case reports of patients with either infections or trauma. [2] In normal joint growth, the condyles are adaptive to variable forces produced by differences in jaw morphology and muscle function. [3] This can result in quite variable changes in length, area and orientation when jaw growth is imbalanced or disproportionate. [4] When transverse skeletal or dental imbalances develop, the condyles adapt by not obtaining normal growth in size, especially in the medio-lateral dimension. [5] These transverse adaptations are reportedly more at risk for development of condyle displacement within the joint and temporomandibular joint disorders (TMD). [6–8]

Dentofacial deformity patients develop the most disproportionate skeletal variations of normal growth and are most likely to have TMJ dysfunction and symptoms. [9] Orthodontic and orthognathic surgical treatments have recently been documented as effective therapies in restoring facial balance and relieving TMD signs and symptoms, especially for related arthralgia or myalgia. [10,11] TMD is also more likely to be associated with dentofacial deformities when a component of the malocclusion involves a significant imbalance in facial symmetry. [12] Well know arthritic conditions like idiopathic condylar resorption may produce skeletal malocclusions and TMD, but in most dentofacial deformity patients condylar modeling is more subtle, and therefore not always considered in treatment planning and outcomes. We recently developed a method for measuring normal condyle geometry variations in a group of patients with dentofacial deformities which revealed differences in condylar length or area between left and right sides. [13] Through genetic analysis we identified a genetic variant in the *ENPP1* gene (rs937300) which associated with these variations as a potential causal factor, since it functions as an inhibitor of hydroxyapatite formation during mineralization. The finding indicates that some individuals may be more susceptible to condyle modeling due to both functional influences and inherent quality of bone adaptation.

When craniofacial asymmetry was present, these patients reported a significantly elevated level of pain and jaw dysfunction. [14] This coincided with significantly elevated clinical diagnosis of disc displacement with reduction, myalgia, arthralgia and TMD related headache. In discriminating between different patterns of asymmetry, we developed a new posterior anterior cephalometric analysis which distinguishes four anatomic subclassifications (group one—four), each with a different rate of TMD symptoms. The mandibular asymmetry categories are described in the Materials and Methods, Section 2.3. In group three, chin deviation is displaced to the side of the face which also has the longer ramus length. This unusual subclassification of asymmetry is very common and results in the highest rate of patient reported TMD symptoms. [14] Genetic analysis revealed that an additional variant in *ENPP1* (rs858339) associated with this asymmetry pattern. Group two and three had the highest rates of reported TMD symptoms, and four had the lowest—even though skeletal imbalance was the most pronounced in this group. Therefore the posterior anterior cephalometric classification of asymmetry may indicate which groups are at higher risk for having or developing TMD, but does not discern which individuals within a group are predisposed. Although other predisposing factors such as

variations in the functional environment are arguably a primary factor influencing TMD, two possible explanations could be differences in condyle geometry variation, during or after growth, and genotype.

Since mandibular morphology is a heritable trait, it is important to consider genetic and epigenetic (functional) influences upon condylar growth and adaptation. [15,16] Fibroblast growth factor 2 (FGF-2) is a primary growth promoter of condylar cartilage growth during development. [17] In animal models where lateral functional shift of the mandible are introduced, condylar FGF-2 expression is increased on the protruded ramus side and decreased on the contralateral retrusive side, introducing asymmetric changes in chondrocyte activity and cartilage morphology. [18] FGF-2 promotes ENPP1 activity, resulting in enhanced subcondral bone mineralization. [19] *ENPP1* has at least 66 functional variants, some of which might respond differently to condylar environmental influences. [20] Therefore, changes in left versus right condyle morphology demonstrated in condyle geometry variation could be the result of developing facial asymmetry during growth, rather than the primary cause. An additional influence on ENPP1 expression is *ACTN3* genotype. In Actn3-/- mice *ENPP1* gene expression is increased, resulting in lowered limb bone mineralization apposition rate, trabecular number and bone volume. [21] We recently associated the common *ACTN3* R577X mutation which results in lack of protein expression, with skeletal Class II malocclusion. The initial hypothesis is the lack of ACTN3 protein results in diminished subcondral bone growth or maintenance through increased ENPP1 activity. [22]

To further understand variation in presentation of TMD signs and symptoms, we evaluated how different patterns of craniofacial asymmetry, asymmetric condyle geometry variation and *ENPP1* or *ACTN3* genotypes might interact, in a cohort of dentofacial deformities subjects already included in previous studies. [13,14,22,23] These findings may be of diagnostic predictive value in counseling patients for their potential risk for developing or aggravation of TMD.

## 2. Materials and methods

### 2.1 Subjects

Subjects with dentofacial deformities who were undergoing elective orthognathic surgery for correction of dento-maxillo-facial dysmorphology (normal variations in jaw geometry which produce malocclusion and facial imbalance) were recruited for study from the Department of Oral and Maxillofacial Surgery, Roger Salengro Hospital, Lille France, after signing an informed consent to participate. The clinic serves an area of northern France of about 4 million inhabitants under the country's National Health Service, and is the region's primary center for maxillofacial surgery. The population for recruitment were non-growing adolescents or adults with a mean age of 26 years and 76% female. They were undergoing combined orthodontic and surgical treatments which included pre-surgical orthodontics, at least a mandibular bilateral sagittal split osteotomy, in conjunction with Lefort osteotomies of the midface as necessary, and a second round of post-surgical orthodontics to finalize occlusion. The study included subjects without other systemic conditions, and excluded those undergoing surgery for facial trauma, tumor, condylar hypertrophy or idiopathic resorption, rheumatoid or osteoarthritis, and congenital craniofacial syndromes or developmental conditions that might influence craniofacial growth. [24] Clinical diagnoses of each patient were summarized at the time of surgery to include the sagittal and vertical malocclusion classification, based upon the extent of required sagittal, vertical and transverse repositioning of jaws estimated in the surgical treatment plan. De-identified information for study included radiographic and diagnostic images, calibrated for magnification, details of the surgery along with information for height, weight, race, ethnicity, age and sex. Subjects signed an informed consent form, and the research

protocol was validated by the French independent ethical committee (Certificate CPP12/44), the Temple University Temple (Certificate 13438) and the University of Pittsburgh institutional review boards (Certificate PRO12080373).

## 2.2 Condyle geometry variation assessment

Although there is no widely accepted method to assess condyle modeling as part of normal growth or physiologic adaptation after maturation, the metric method (two dimensional radiographic measurements) have historically been utilized. [25,26] In our patient population, we recently developed a metric measurement method that compares morphometric differences between left and right condyle height or condyle area on panoramic radiographs. [13] Two lines were constructed to evaluate condylar height, one drawn tangential to the posterior edge of the mandible passing through the most posterior points of the condyle and mandibular ramus, and the perpendicular line passing through the lower end of the mandibular notch. Condylar height was measured perpendicular to the latter between the mandibular notch and the highest point of the condylar unit (Fig 1A). The surface of the condylar unit was measured, contouring the lowest point of the mandibular notch to the *lingula mandibulae*, then perpendicular to the rear edge of the mandibular ramus (Fig 1B). Bone modeling was determined by a differential measurement of condylar height or condylar surface defined by a percentage in relation to the larger side between right and left sides on a pre-surgical panoramic radiograph. From this patient data we were able to associate a genetic variant in *ENPP1* with mandibular condyle geometry variation. [13] For comparison, we utilized this existing data base, in combination with an assessment of craniofacial asymmetry to determine associations with TMD or genetic variations. Differences greater than 3% between sides were considered positive for condyle modeling and recorded as percentage difference between sides. Differences less than 3% were recorded as no difference between sides or 0%. Landmarks have been defined on

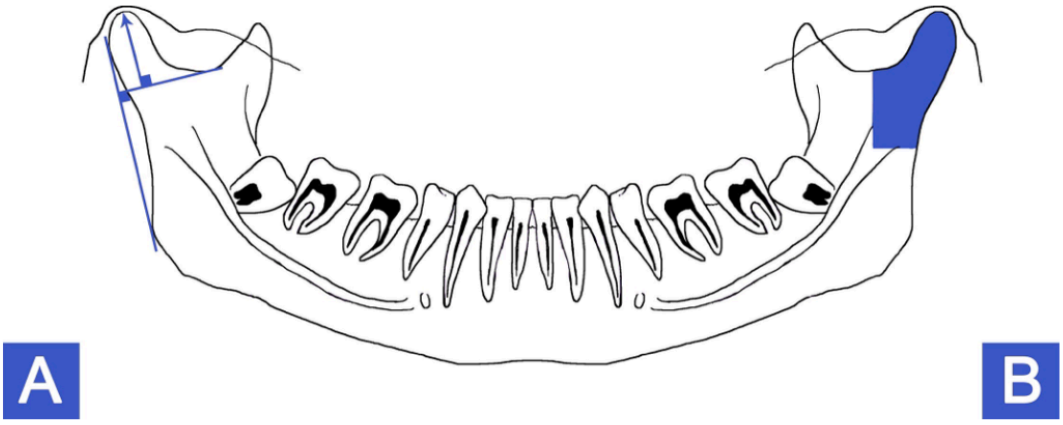


**Fig 1. Panoramic landmarks related to condyle modelling measurements.** A–Two lines were constructed to evaluate condylar height, one drawn tangential to the posterior edge of the mandible passing through the most posterior points of the condyle and mandibular ramus, and the perpendicular line passing through the lower end of the mandibular notch. Condylar height was measured perpendicular to the latter between the mandibular notch and the highest point of the condylar unit. B—The surface of the condylar unit was measured, contouring the lowest point of the mandibular notch to the *lingula mandibulae*, then perpendicular to the rear edge of the mandibular ramus. Bone modeling was determined by a differential measurement of condylar height or condylar surface defined by a percentage in relation to the larger side between right and left sides on a pre-surgical panoramic radiograph.

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calibrated radiographs, using a cephalostat. Data acquisition has been performed by two observers, jointly defining the landmarks. All measures were done using ImageJ software (National Institute of Health, Bethesda, MD, USA).

### 2.3 Asymmetry assessment and classification

Craniofacial asymmetry is a type of dentofacial deformity which has a unique set of morphologic variations for which there have been many classification approaches. We recently developed a new diagnostic assessment based upon 17 anatomic landmarks on posteroanterior cephalometric radiographs. [14] These landmarks were converted into 6 cephalometric metric assessments which characterized four different asymmetry subtypes present in the population (Fig 2). We characterized these as group 1: asymmetry of the mandibular body, but symmetry in mandibular rami (sometimes termed "mandibular yaw"); group 2: differences in left and right ramus heights, with mandibular chin deviated towards the shorter ramus height side (what most clinicians would refer to as a typical facial asymmetry); group 3: differences in ramus heights with mandibular chin point deviated towards the longer ramus height side (an "atypical" facial asymmetry); and group 4; differences in left and right ramus heights, with mandibular chin deviated towards the shorter ramus height side (as with group 2) but in addition with pronounced maxillary midfacial canting. From the cephalometric analysis patients were classified as symmetric or asymmetric, and if asymmetric into subtypes. From these patient groupings we previously found asymmetry group 2 and 3 had the highest incidence of pre-surgical TMD, and groups 1, 3 and 4 had significant associations with genetic variants in *ENPP1* [14]. In the present study we compared these classifications for asymmetry to differences on condyle geometry, as determined in section 2.2.

### 2.4 Assessment of temporomandibular disorders

Temporomandibular joint functioning was assessed as a routine part of the pre-surgical evaluation using the Diagnostic Criteria for TMD (DC/TMD). [27] Overall this young population is not presenting with fibromyalgia or pain related disability diagnosed in Axis II of the diagnostic criteria. The three common Axis I disorders associated with asymmetry in the population were disc displacement with reduction (DDR) (78%), myalgia (61%) and arthralgia (33%). [14] We use the jaw pain and function (JPF) questionnaire to assess patient reported symptoms as an indication of perceived severity before and one year after jaw surgery. [23] The JPF was developed as a simple screening tool to determine presence of TMD. [28] It consists of eight questions about jaw pain and five questions related to jaw function. The questionnaire has been validated to reliably distinguish between normal (scores $< 6$) and TMD subjects (scores $\geq 6$) with up to 98% sensitivity and 100% specificity. [29] It has been validated in European translations [30] and we use a French version. [14,23] In this assessment, we included TMD patients with positive diagnosis for DDR, myalgia and/or arthralgia. Patients with positive clinical diagnosis for other, less common forms of TMD were excluded from study since they were insufficient number to investigate.

### 2.5 Comparing condyle variation with facial asymmetry

A total of 128 subjects had complete data sets for comparison of condyle variation with symmetry classification. We compared condyle height or condyle surface as percent differences between sides, and which side, either left or right, was longer or larger to the symmetry classification of patients. Symmetric subjects and those in asymmetry group 1 had equal left and right mandibular ramus length. In the other three asymmetry groups one ramus was larger in length and one smaller. In groups 2 and 4 the chin, as indicated by the mandibular menton landmark,

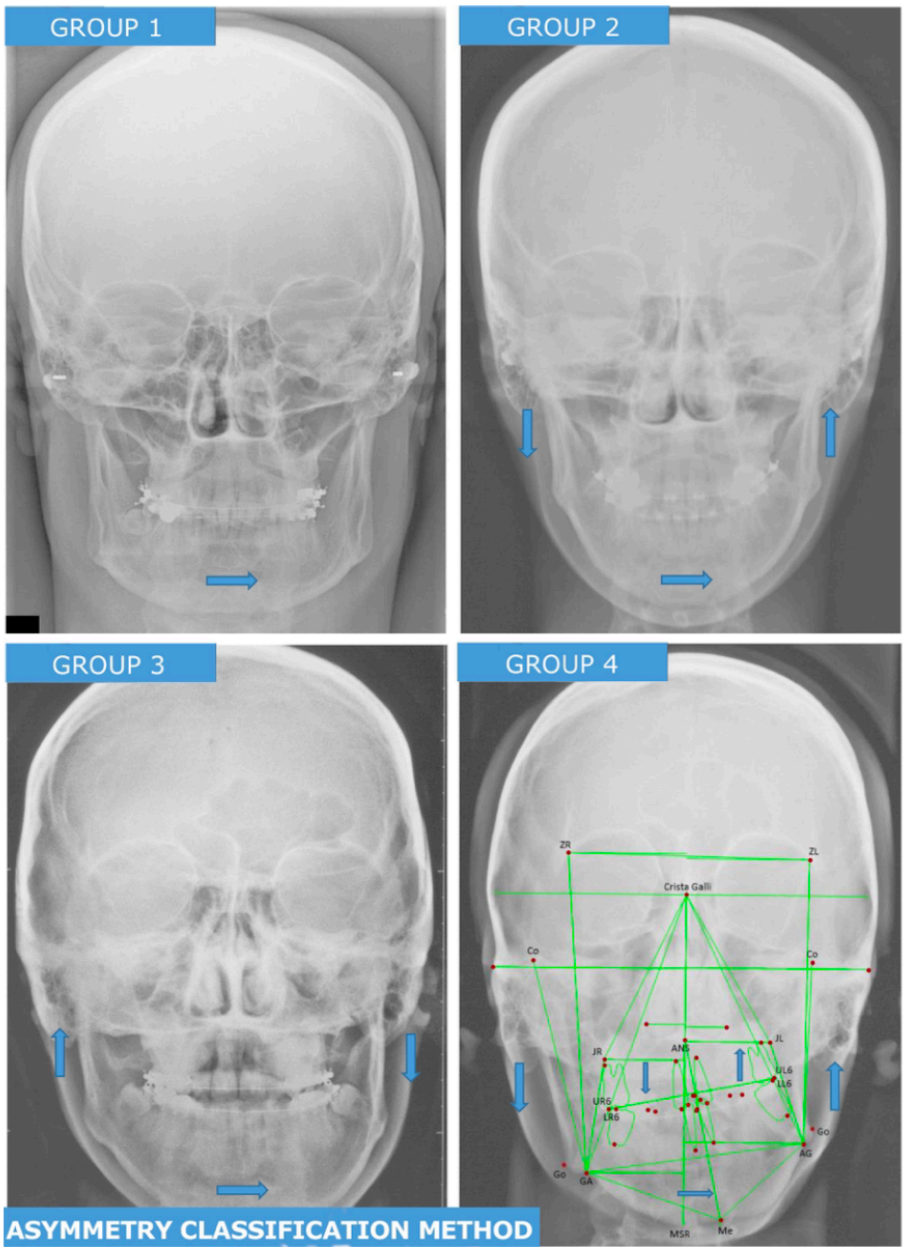
GROUP 1
GROUP 2
GROUP 3
GROUP 4
ZR
ZL
Crista Galli
Co
Co
JR
AM6
JL
UR6
LR6
UL6
LL6
Go
Go
GA
AG
MSR
Me
ASYMMETRY CLASSIFICATION METHOD

**Fig 2. Prototypes for four asymmetry subtypes and illustration of PA cephalometric tracing.** Group 1—mandibular body asymmetry, but symmetry in mandibular rami (sometimes termed "mandibular yaw"); Group 2—ramus asymmetry: differences in left and right ramus heights with mandibular chin deviated towards the shorter ramus height side; Group 3—atypical asymmetry: differences in ramus heights with mandibular chin point deviated towards the longer ramus height side; and Group 4—C-shaped asymmetry: differences in left and right ramus heights, with mandibular chin deviated towards the shorter ramus height side (as with group 2) but in addition with pronounced maxillary midfacial canting. Landmarks used for cephalometric analysis labeled on Group 4.

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was deviated away from the facial midline towards the shorter ramus length side. In group 3 the chin however was deviated away from the facial midline towards the longer ramus length side.

In comparing condyle differences to these patterns of chin and ramus asymmetry, we anticipated finding that the longer or larger condyle would be located on the same side as the longer ramus. However, this was not true in the majority of patients. Rather, it was more likely that increased condyle dimension was located on the side with the shorter ramus dimension. Because of this unexpected finding, we further classified asymmetry groups into those who followed the normal, expected pattern or those with an unexpected pattern as follows: (Table 1).

This criteria recognizes that in asymmetry group 3 the menton is deviated towards the shorter ramus side, but since the ramus is longer on this side, it is anticipated that condyle dimension would also be larger. Figs 3 and 4 compare of one subject in group 3 which had normal condyle modeling (1) and one with abnormal modeling (2).

Based upon this realization the anatomical investigation to study had two primary endpoints: 1) determine the frequency of condyle variation in patients with symmetry compared to asymmetry and 2) determine if condyle variation could have contributed to differences in TMD, in the differing patterns of asymmetry.

## 2.6 Genotyping

Saliva samples were collected during the pre-surgical evaluation and processed utilizing DNA Genotek kits. Genomic DNA was used for profiling of polymorphisms using TaqMan chemistry [31] and for sequencing using an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems). Seven single nucleotide polymorphisms were selected if genotyping: in *ACTN3* rs1671064, rs1815739 and rs678397 [22] and in *ENPP1* rs937300, rs6569759, rs858339, and rs1409181. [23] The asymmetry population was compared for SNP variants between normal versus abnormal modeling, as summarized in Table 1, section 2.5.

## 2.7 Statistical testing

Differences in condyle height or condyle area were compared between all symmetric and all asymmetric subjects using an unpaired *t* test, and an ANOVA for comparison between the different asymmetry groups. For relationship to TMD, JPF scores were compared for each individual asymmetry group between normal growth and abnormal growth by individual unpaired *t* tests. In cases where individual asymmetry group comparisons revealed no significant differences for JPF, all asymmetry groups were averaged together (normal vs. abnormal

**Table 1. Criteria for normal vs. abnormal condyle variation in asymmetry condyle height or area difference.**

| same side as menton deviation | opposite side as menton deviation |
|---|---|
| Group 1—abnormal pattern | Group 1—normal pattern |
| Group 2—abnormal | Group 2—normal |
| Group 3—normal | Group 3—abnormal |
| Group 4—abnormal | Group 4—normal |

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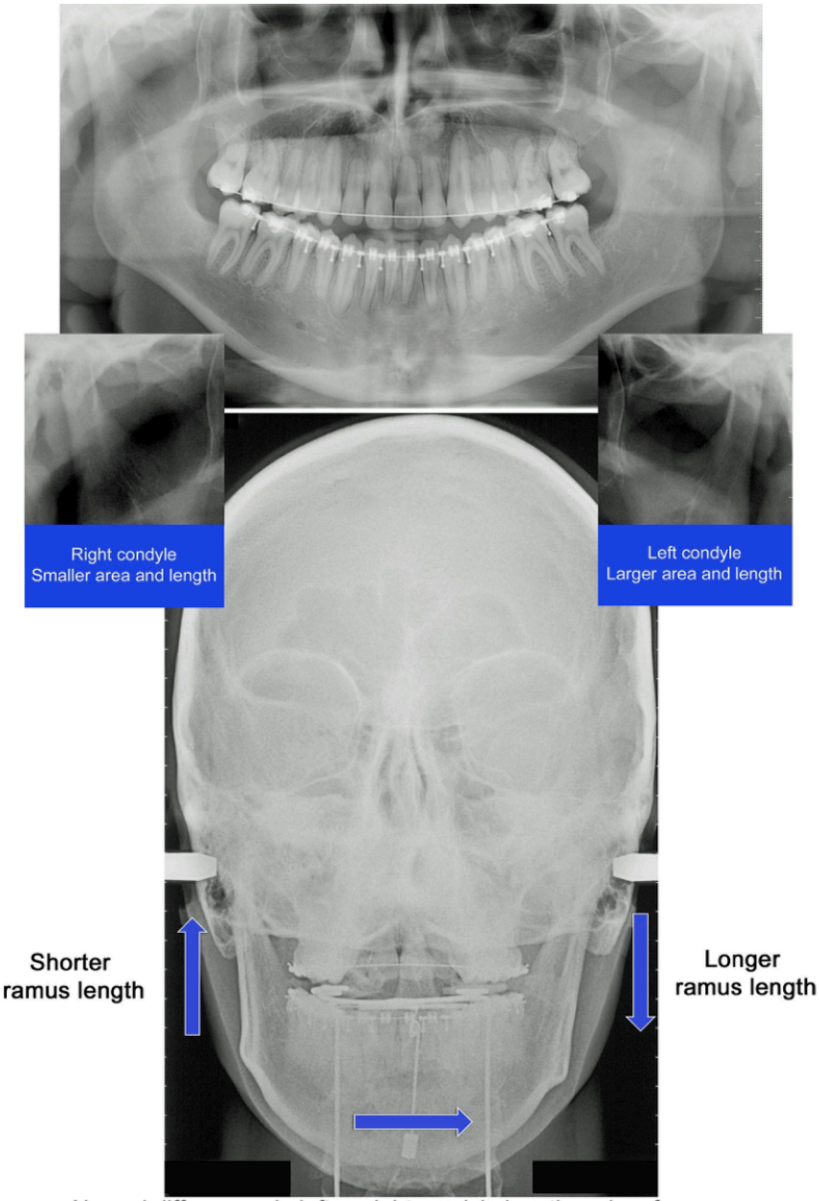



Normal differences in left vs right condyle length and surface area
For asymmetry group 3

**Fig 3. Illustration of normal condyle modeling.** Subject from asymmetry group 3, atypical asymmetry, with menton deviated toward longer ramus side. Condyle geometry variation defined as "normal" due to longer and larger condyle on side with longer ramus length.

https://doi.org/10.1371/journal.pone.0236425.g003

condyle height and area), and compared by Student *t* tests to determine significance. For clinical diagnoses of TMD, Chi-square tests were used to compare individual and all TMD diagnoses between individual asymmetry groups, and for the number of TMD diagnosis between all normal vs. abnormal condyle height and area groups. Chi-square and Fisher's exact tests were used to determine the over-representation of genotypes and alleles.

## 3. Results

### 3.1 Differences in condyle modeling between symmetry and asymmetry

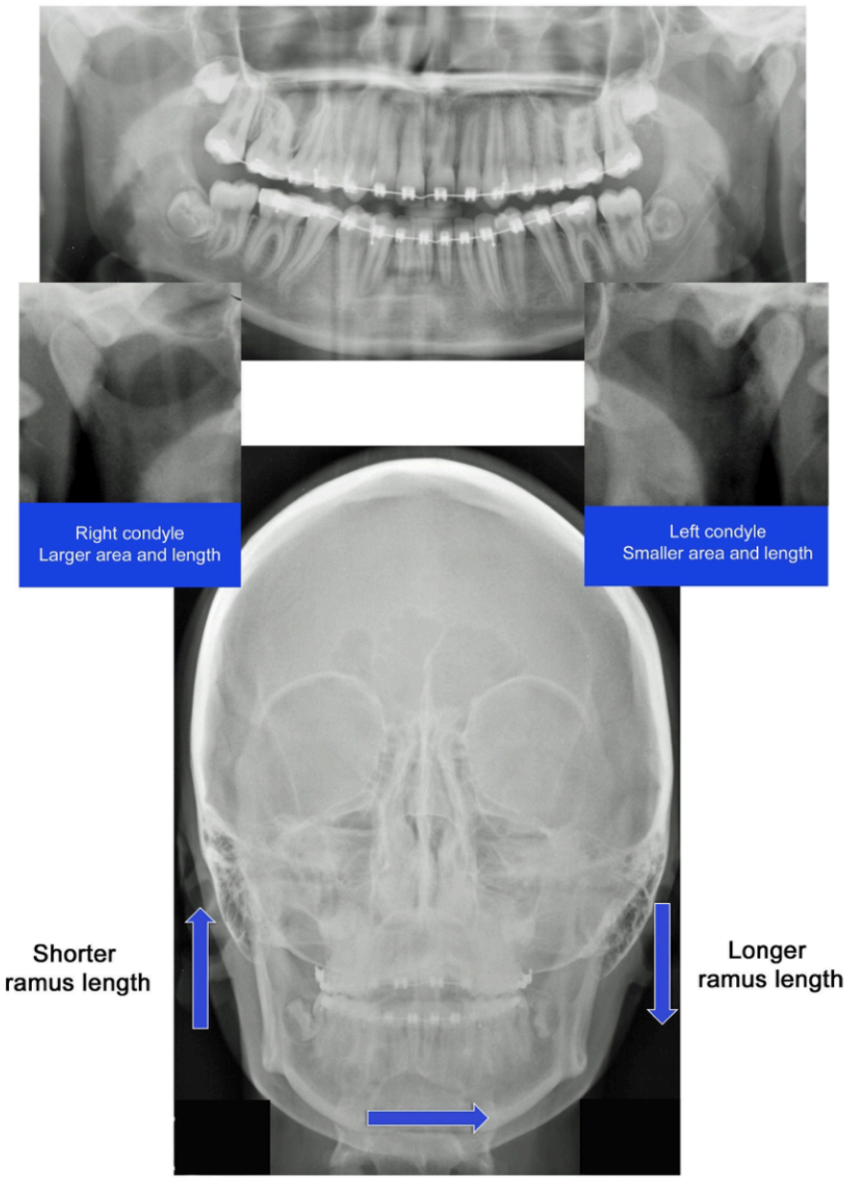
Complete data was available from 128 subjects, 56 were classified within one of four craniofacial asymmetry groups from posterior anterior cephalometric analysis. When compared for differential bone modeling of mandibular condyles from panoramic radiographic analysis, there were very significant differences between symmetric vs asymmetric subjects. In the symmetric group there was a mean condyle height variation between sides of 7.37% ± 5.49, compared to 10.89% ± 7.39 for the asymmetric group, which was significantly different ($p = 0.0025$). The condyle area mean difference in symmetrics was 8.22% ± 5.53, while the asymmetric group difference was 10.35% ± 8.35, which was nearly significantly different at ($p = 0.08$).

Within the asymmetry population individual groups were compared to determine if there was a difference in the amount of left compared to right condyle height or area modeling. There was no significant differences for condyle height, but there was a significant difference for condyle area ($p = 0.02$). For condyle area, the amount of difference between sides increased as the severity of asymmetry became more pronounced. Group 1 had a mean area difference of 4.41%, group 2–11.62%, group 3–14.17% and group 4–16.56%.

### 3.2 TMD differences in asymmetries between normal and abnormal condyle modeling

We investigated whether normal vs abnormal condyle growth modeling, based upon study criteria (Table 1), might contribute to differences in TMD prevalence. This revealed that abnormal condyle modeling was the most common finding throughout the classifications of asymmetry (Table 2). For condylar height, abnormal modeling ranged from 50 to 70 percent. For condylar area, modeling rates were higher in most groups, at rates between 50 and 75. Only asymmetry group 2 had and almost equal distribution of normal versus abnormal modeling.

We compared patient reported TMD symptoms using the JPF questionnaire between normal and abnormal condyle height or area, for each asymmetry group. The abnormal condyle height group had a mean JPF score or 6.3 while the normal group score was 4.7, demonstrating an almost significant difference in symptoms $p = 0.055$. Those with abnormal condyle area reported mean JPF score of 5.8 compared to the normal area group score of 5.5, resulting in no significant difference $p = 0.75$. When abnormal condyle height or area were grouped together for the entire population, there was an elevation in mean JPF score to 6.65 by comparison to 5.33 in normal modeling group ($p = 0.023$). Patient symptoms were greater in asymmetry groups 2 and 3, and either abnormal height or areas contributed to pain and functional differences at approximately the same rate.




Abnormal differences in left vs right condyle length and surface area
For asymmetry group 3

**Fig 4. Illustration of abnormal condyle modeling.** Subject from asymmetry group 3, atypical asymmetry, with menton deviated toward longer ramus side. Condyle geometry variation defined as "abnormal" due to longer and larger condyle on side with shorter ramus length.

https://doi.org/10.1371/journal.pone.0236425.g004

Clinical diagnosis of TMD was compared between normal and abnormal modeling for the population. Since multiple positive diagnoses were common, we first assessed if TMD was present or absent, regardless of single or multiple diagnoses in each patient, which we called "all TMD" patients. This overall positive versus negative comparison revealed a strong trend with a relative higher prevalence of 'all TMD" in the abnormal group ($p = 0.05$) (Table 3). In individual TMD diagnoses of headache, myalgia, arthralgia, disc displacement with reduction and disc displacement without reduction there were no significant differences. However, there was a trend for diagnosis of myalgia ($p = 0.06$). When total, multiple individual diagnoses were grouped for overall comparison, there was affirming data that abnormal condyle modeling resulted in increased problems with TMD ($p = 0$ .000001).

### 3.3 Genotype differences

For genotype comparisons we grouped all abnormal condyle height and abnormal condyle area modeling subjects together and compared them to those with normal modeling in each individual asymmetry group. For *ENPP1* there were no significant differences in genotypes or alleles for SNPs rs937300 ($p$ range values 0.29 to 0.88), rs6569759 (p range values 0.85 to 0.92) or rs858339 (p range values 0.37 to 0.51). For *ACTN3* however there were significant differences in group 2 for genotypes rs1671064 ($p = 0.02$), rs678397 ($p = 0.04$) and alleles 1815739 ($p = 0.00$) (Table 4). There was a trend for rs1815739 genotypes ($p = 0.08$). Results were most likely positive in group 2 since it had the most subjects for comparison.

## 4. Discussion

### 4.1 Condylar role in mandibular modeling

Modeling is the process by which bone enlarges and takes shape during normal growth. Modeling is a complimentary process of resorption or deposition to generate new tissue during homeostasis or for modification of size and shape. The condyle contributes to both mandibular modeling and modeling during normal growth, and has inherent capacity to remodel after growth is completed through chondrocyte sensitivity to variations in mechanotransduction. [32] These effects are well documented in orthodontic treatment of Class II malocclusions with repositioning appliances, where condyles enlarge in anterior-posterior dimension, compared to untreated controls in adolescent or even young adult patients. Ramus modeling can also occur at the same time as changes in condyle dimensions. [33] This ability to adapt and transition from chondrogenesis to osteogenesis is a unique anabolic feature of condylar

**Table 2. Condyle modeling proportions in asymmetries.**

| | height modeling | | | | area modeling | | | |
|---|---|---|---|---|---|---|---|---|
| | abnormal | | normal | | abnormal | | normal | |
| | n | % | n | % | n | % | n | % |
| asymmetry group 1 | 5 | 62.5 | 3 | 37.5 | 6 | 75 | 2 | 25 |
| asymmetry group 2 | 11 | 48 | 12 | 52 | 12 | 52 | 11 | 48 |
| asymmetry group 3 | 12 | 66.6 | 6 | 33.3 | 13 | 72 | 5 | 28 |
| asymmetry group 4 | 5 | 71 | 2 | 29 | 4 | 57 | 3 | 43 |

https://doi.org/10.1371/journal.pone.0236425.t002

**Table 3. Differences in TMD diagnosis between normal and abnormal condyle modeling.**

| Condyle | group 1 | | group 2 | | group 3 | | group 4 | | total | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| area + height | abnormal (11)* | normal (5) | abnormal (23) | normal (23) | abnormal (25) | normal (11) | abnormal (9) | normal (4) | abnormal (68) | normal (43) | Chi-squared test |
| all TMD | 3 | 1 | 13 | 8 | 11 | 3 | 1 | 0 | 28 | 10 | $p = 0.05$ |
| headache | 0 | 0 | 3 | 1 | 2 | 0 | 1 | 1 | 6 | 2 | $p = 0.41$ |
| myalgia | 3 | 1 | 8 | 5 | 15 | 2 | 1 | 2 | 27 | 10 | $p = 0.06$ |
| arthralgia | 0 | 0 | 3 | 4 | 6 | 4 | 0 | 0 | 9 | 8 | $p = 0.42$ |
| DDR | 2 | 0 | 9 | 5 | 15 | 6 | 1 | 1 | 27 | 12 | $p = 0.19$ |
| DD w/o R | 0 | 0 | 2 | 0 | 5 | 1 | 0 | 0 | 7 | 1 | $p = 0.11$ |
| total positive TMD Diagnoses (including multiple diagnosis for each subject) | | | | | | | | | 68 | 33 | $p = 0$ .000001 |

*(n) indicates total number of subjects per group; DDR = Disc displacement with reduction; DD w/o R = Disc displacement without reduction without limited opening.

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cartilage. In environments where forces differ in the transverse occlusal plane due to crossbite, some reports have identified asymmetrical condylar modeling, while other have not. In most studies considering skeletal asymmetries of the mandible, condyles are reported to be asymmetric, with decreased cross sectional area, surface size and ramal height on the deviated side. [34,35]

As descriptive morphology of craniofacial asymmetries has advanced, several classification approaches which emphasize mandibular roll, pitch and yaw have established that ramus length, menton deviation and condylar morphological variations do not always match each other, and ramus height may be longer on the same facial side to menton deviation. [14] We recently developed a posterior–anterior celphalometric analysis utilizing six measurements, with four of these detecting mandibular differences between the body, width, ramus length and menton deviation. When viewed by principal component analysis, symmetric and asymmetric faces cluster as distinct groups. Variability in asymmetric groups 1–4 revealed that principal components clustered by differences between the left and right mandibular sides, indicating that a consistent geometric variability explained differences in morphology between them. [14] The main fluctuating variable between groups is the relationship of chin deviation in the transverse plane to left vs right ramus length differences between sides. With regard to these relationships, this study has demonstrated two distinct patterns of asymmetry which have not been commonly recognized previously. First, it is almost equally common to have longer ramus length on the same side as chin deviation as it is to have a longer ramus on the contralateral side. Second, the condyle on the longer ramus side may be geometrically smaller than on the shorter ramus side, regardless of the specific menton to ramus relationship. Insight into these different patterns of

**Table 4. Comparison of SNP genotypes by condyle modeling pattern.**

| Gene | ACTN3 | rs1671064 (Q523R) | | | p value | rs1815739 (R577X) | | | p value | rs678397 (intronic) | | | p value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP | | GG | GA | AA | genotype/allele | TT | CC | TC | genotype/allele | TT | CC | CT | genotype/allele |
| Group 1 | normal | 2 (15) | 7 (54) | 4 (31) | $p = 0.15$/ 0.08 | 2 (15) | 4 (31) | 7 (54) | p = 0.11/ 0.07 | 2 (15) | 4 (31) | 7 (54) | $p = 0.15$/ 0.07 |
| | abnormal | 2 (66) | 1 (33) | 0 | | 2 (66) | 0 | 1 (33) | | 2 (66) | 0 | 1 (33) | |
| Group 2 | normal | 5 (22) | 18 (78) | 0 | $p =$ **0.02**/ 0.08 | 9 (33) | 1 (4) | 17 (63) | p = 0.08/ **0.00** | 10 (40) | 1 (4) | 14 (56) | $p =$ **0.04**/ 0.08 |
| | abnormal | 5 (26) | 9 (48) | 5 (26) | | 5 (26) | 6 (32) | 8 (42) | | 6 (31) | 6 (31) | 7 (38) | |
| Group 3 | normal | 2 (8) | 14 (61) | 7 (31) | $p = 0.69$/ 0.49 | 2 (4) | 6 (33) | 16 (63) | p = 0.84/ 0.34 | 2 (10) | 7 (33) | 12 (57) | $p = 0.72$/ 0.64 |
| | abnormal | 2 (12) | 11 (69) | 3 (19) | | 2 (12) | 3 (19) | 11 (69) | | 2 (15) | 3 (21) | 9 (64) | |
| Group 4 | normal | 4 (40) | 6 (60) | 0 | $p = 0.73$/ 0.79 | 4 (40) | 0 | 6 (60) | p = 0.3/ 0.79 | 4 (40) | 2 (20) | 4 (40) | $p = 0.62$/ 0.45 |
| | abnormal | 2 (50) | 2 (50) | 0 | | 2 (50) | 0 | 2 (50) | | 2 (50) | 0 | 2 (50) | |

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asymmetry most likely arise from the number of subjects we have been able to evaluate and compare, rather than previous studies with a more limited population from which these more subtle variations would be less possible to distinguish. These findings raise important clinical questions in patient diagnosis, treatment and management, since differences may relate to intrinsic genetic factors and risk of pre or post treatment TMD and stability.

### 4.2 Condyle modeling and signs or symptoms of TMD

In our population, asymmetric subjects have both higher clinical diagnoses of TMD and higher reported TMD symptoms, as indicated by the JPF survey. [14] Yet within individual asymmetric groups, the standard deviation for mean values of patient reported symptoms are quite high. This is especially true for group three which had a mean JPF score of $9.11 \pm 5.62$. Likewise group two, also with elevated symptoms had JPF score of $6.94 \pm 5.46$. Therefore we evaluated if the pattern of condyle modeling might influence symptom variability by comparing expected versus unexpected geometry variations (Table 1). This resulted in a significantly elevated ($p = 0.02$) level of patient reported symptoms when modeling did not match with ramus height or area differences. For TMD diagnosis the same comparison revealed a significant difference for subjects with at least one positive finding ($p = 0.05$) (Table 3). While the individual types of TMD were not significant, when the total number of patient diagnoses were compared, there was very significantly elevated differences ($p = 0.0001$). This resulted since those with abnormal condyle modeling most often had multiple combinations of different types of TMD. The most common TMD diagnoses in both normal and abnormal groups were masticatory muscle myalgia and disc displacement with reduction, with abnormals almost three times more likely to have myalgia and twice as likely to have disc displacement. Arthralgia rates were almost equal between groups. In abnormal modeling arthralgia was not nearly as likely, with a ratio of 3:1 compared to myalgia. Since presence of arthritic conditions or condylar resorption were part of the study exclusion criteria, the study considered if differences in condylar modeling produced different rates of arthralgia, and found no difference.

An opposite finding occurred for myalgia and disc displacement with reduction, which are much more likely with abnormal condyle modeling. Myalgia was the only individual TMD diagnosis which was almost significantly different for condyle modeling ($p = 0.06$), and emerged as the solitary clinical diagnosis most related to abnormal modeling (Table 3). The asymmetric patients under study had significant skeletal imbalances, when evaluated by posterior-anterior cephalometric analysis, with the greatest skeletal variation being ramal height differences at $p < 0.0001$. [14] Muscle functioning is also imbalanced in craniofacial asymmetry, with a significant increase in fast twitch skeletal muscle fibers on the side to which the menton was deviated, when analyzed from masseter muscle biopsy during surgery. [14] Imbalanced force during whole muscle contraction in repetitive athletic activity is a well-known cause of myalgia, especially in the lower back and shoulder. Women athletes with hip strength asymmetry, a type of hip muscle imbalance, are more likely to develop occurrences of low back pain. [36] For male wheelchair athletes, weakness in humeral head depressors, a shoulder muscle imbalance, can result in development of rotator cuff impingement syndrome. Muscle rather than joint pain may also arise from repetitive, unvaried, continuous locomotion and often affect women more than men in the upper extremities. [37]

The TMJ is a unique craniofacial joint since there are three articulations, each joint and the occlusion. Postural and functional position of the jaws is coordinated by afferent input from muscle spindles throughout the head and neck and imbalances in the trigeminal motor system can produce imbalanced stress distribution throughout the cervical spine. [38] Facial skeletal asymmetry produces functional imbalances in masticatory muscles with greater activation on

the longer ramus side. This produces an uneven stress distribution in the mandible, which may occur due to either differences in masticatory muscle forces or skeletal geometry. [38] The TMJ can buffer imbalanced mechanical stress by alteration in rates of chondrogenesis, which may be the etiology of abnormal condyle modeling. In animal models, imbalance in masticatory muscle activity results in asymmetric growth of subcondral bone to normalize stress distributions. [39] This presents the interesting possibility that individual patients adapt better to craniofacial asymmetry if condyle geometry differences provide positive stress support within the joint. Imbalances may also explain why patients experience high rates of myalgia, since increased peripheral activation of masseter muscle spindles can contribute to and help maintain chronic muscle pain. [40]

### 4.3 *ACTN3* anpd *ENPP1* genotypes

ENPP1 is a trans membrane glycoprotein which synthesizes inorganic phosphate from extracellular ATP, inhibiting hydroxyapatite formation. SNPs in *ENPP1* are associated with a large number of bone diseases and abnormal bone and joint morphology. Different mechanical strain environments change *ENPP1* expression which can lead to either protection or calcification of endplate cartilage chondrocytes. [41] In the mandible we recently found the rs937300 SNP associated with variation in condyle geometry between left and right sides. [13] The GG genotype was protective against condyle height reduction. Therefore it is likely that both genotype and functional variations contribute to the pattern of condyle modeling. Two SNPs also associated with either group 1 or group 3 craniofacial asymmetry. [14] Therefore, we evaluated the possibility that *ENPP1* variants also contributed to abnormal condyle modeling, but have so far found no associations.

ENPP1 and ACTN3 are connected in bone adaptation by an unknown biologic mechanism. Nevertheless, in Actn3-/- mice osteoblasts have up-regulated expression of ENPP1 which may lead to differences in mineralization rates. [21] This *in vitro* connection is consistent with disruption of normal mineralization resulting in an overall decrease in bone mass in α-actinin-3 deficiency. In humans the *ACTN3* R577X (rs1815739) null polymorphism associates with higher serum levels of modeling markers, which may make bone more susceptible to geometry variations. [42] We found a very significant association ($p < 0.0001$) for R577X allele differences, with the X allele (null polymorphism) elevated in the normal condyle modeling group. There were additional significant associations for *ACTN3* rs1671064 and rs678397 genotypes. rs1671064 is the Q523R polymorphism which produces an A to G transition not know to have functional consequences. Q523R however has been found to have linkage disequilibrium with R577X, [43] and this may indicate that R577X being tested is in close proximity (in linkage disequilibrium) with Q523R, which we could speculate is the actual genetic variant (mutation) that is leading to condyle modeling and TMD symptoms. rs678397 is an intronic SNP which has previously been identified as having a very significant association ($p = 0.003$) with skeletal class II malocclusions, most likely through variations in condylar growth. [22] All of these findings indicate that both *ENPP1* and *ACTN3* genotypes associate with varying patterns of condyle modeling in ways which are not yet understood. *ACTN3* genotypes can influence *ENPP1* expression, as can changes in cartilage mechanical strain environments. [40] Differing biomechanical forces as epigenetic factors and intrinsic genetic differences, both contribute to the pattern of condyle modeling stability or instability, and require further investigation.

### 4.4 Study shortcomings

The study used conventional posterior anterior cephalograms and panoramic radiographs to determine morphologic differences in the pattern of craniofacial asymmetry and condyle

modeling. These imaging modalities are routinely utilized in radiographic evaluation of dental patients. Although commuted tomography (CBCT) is more precise, current clinical guidelines from the American Dental Association and American Association and Pediatric Dentistry recommend prescription of panoramic radiographs for routine periodic imaging.

Although the study investigated a relatively large number of patients, the study protocol separated participants almost in half for symmetry, and the asymmetric subjects were eventually sub divided into 8 groups for asymmetry type and condyle remolding differences. This resulted in statistical comparisons between limited numbers of patients between groups, and the most important study shortcoming. Future directions will include ongoing studies with larger subject numbers to further understand how condyle modeling and craniofacial asymmetry arise and interact.

### 4.5 Conclusions

In dentofacial deformity subjects, craniofacial asymmetry, abnormal patterns of condyle modeling and TMD are common comorbidities. Condyle geometry variations between mandibular sides and TMD are more common if asymmetry is present as part of the deformity. Often, asymmetric condyle geometry variation does not match differences in ramus length, found in different classifications of facial asymmetry. TMD signs and symptoms are more likely when condyle variations and ramus asymmetry do not match. The most common TMD diagnosis is masticatory muscle myalgia, which likely results from unequal force distributions. *ACTN3* genotypes under study associate with asymmetric condyle modeling and Q523R (missense) may be in linkage disequilibrium with R577X, the common null polymorphism.

These findings further diagnostic precision for interpreting which individual patients might have or develop TMD with or without symptoms. Those in asymmetry groups two and three with imbalanced condyle geometry variation seem to be most at risk, and this is useful diagnostic information. As patients develop asymmetries during maturation, evaluating these features could be important considerations in patient counseling regarding risks for not undergoing surgical treatment to correct skeletal discrepancies.

## Supporting information

**S1 Data.**
(XLS)

## Author Contributions

**Conceptualization:** Romain Nicot, Alexandre R. Vieira, James J. Sciote.

**Data curation:** Romain Nicot, Kay Chung, Alexandre R. Vieira, Gwénaël Raoul, Joël Ferri.

**Formal analysis:** Romain Nicot, Kay Chung, Alexandre R. Vieira.

**Funding acquisition:** James J. Sciote.

**Investigation:** James J. Sciote.

**Methodology:** Romain Nicot, Alexandre R. Vieira.

**Project administration:** James J. Sciote.

**Supervision:** James J. Sciote.

**Validation:** Romain Nicot, Alexandre R. Vieira, Gwénaël Raoul, Joël Ferri, James J. Sciote.

**Writing – original draft:** Romain Nicot, James J. Sciote.

**Writing – review & editing:** Romain Nicot, Kay Chung, Alexandre R. Vieira, Gwénaël Raoul, Joël Ferri, James J. Sciote.

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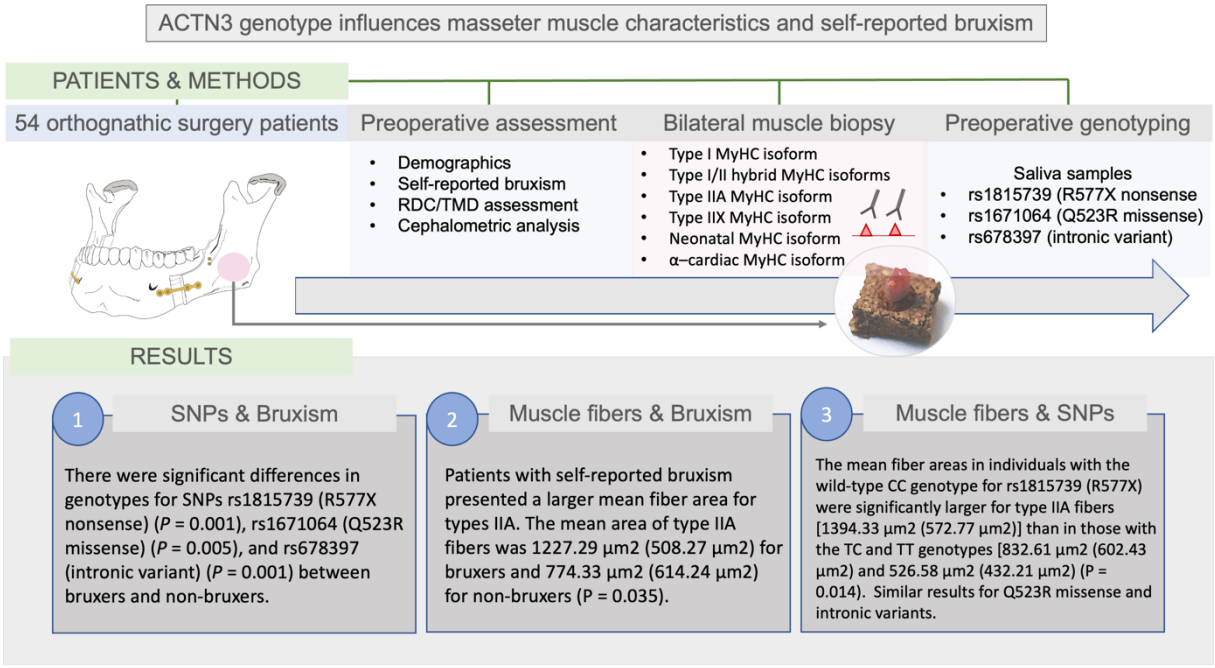
## IV. Génotypes d'*ACTN3*, bruxisme et muscle masséter

INTRUCTION : L'objectif principal de l'étude était d'explorer l'association entre les polymorphismes génétiques d'*ACTN3* et le bruxisme auto-déclaré dans une cohorte de jeunes adultes présentant une dysmorphose dentofaciale.

METHODE : 54 patients pris en charge pour une chirurgie orthognathique dans la prise en charge d'une dysmorphose dentofaciale ont été inclus. Le bruxisme auto-déclaré et le statut dysfonctionnel temporomandibulaire ont été recueillis en préopératoire. Des échantillons de salive préopératoires ont été prélevés pour le génotypage d'*ACTN3*. Des échantillons bilatéraux de muscle masséter ont été prélevés au moment de la chirurgie orthognathique pour l'étude histomorphométrique.

RESULTATS : Il y avait des différences significatives dans les génotypes pour rs1815739 (*R577X* non-sens) (p=0.001), rs1671064 (*Q523R* faux-sens) (p=0.005) et rs678397 (variant intronique) (p=0.001) entre les bruxeurs et les non-bruxeurs. Les patients présentant un bruxisme auto-déclaré présentaient une surface moyenne des fibres musculaires plus importante pour le type IIA (p=0.035). Les surfaces moyennes des fibres chez les individus avec le génotype CC de type sauvage pour rs1815739 (*R577X*) étaient significativement plus grandes pour les fibres de type IIA [1394.33 m2 (572.77 µm2)] que chez ceux avec les génotypes TC et TT [832.61 µm2 (602.43 µm2) et 526.58 m2 (432.21 µm2) (p=0.014). Des résultats similaires ont été retrouvés pour les variants faux-sens et introniques.

CONCLUSION : Le génotype d'*ACTN3* influence le bruxisme autodéclaré par le biais de caractéristiques spécifiques des fibres musculaires masséters.


ACTN3 genotype influences masseter muscle characteristics and self-reported bruxism
PATIENTS & METHODS
54 orthognathic surgery patients
Preoperative assessment
Demographics
Self-reported bruxism
RDC/TMD assessment
Cephalometric analysis
Bilateral muscle biopsy
Type I MyHC isoform
Type I/II hybrid MyHC isoforms
Type IIA MyHC isoform
Type IIX MyHC isoform
Neonatal MyHC isoform
α–cardiac MyHC isoform
Preoperative genotyping
Saliva samples
rs1815739 (R577X nonsense
rs1671064 (Q523R missense)
rs678397 (intronic variant)
RESULTS
1
SNPs & Bruxism
There were significant differences in genotypes for SNPs rs1815739 (R577X nonsense) (P = 0.001), rs1671064 (Q523R missense) (P = 0.005), and rs678397 (intronic variant) (P = 0.001) between bruxers and non-bruxers.
2
Muscle fibers & Bruxism
Patients with self-reported bruxism presented a larger mean fiber area for types IIA. The mean area of type IIA fibers was 1227.29 µm2 (508.27 µm2) for bruxers and 774.33 µm2 (614.24 µm2) for non-bruxers (P = 0.035).
3
Muscle fibers & SNPs
The mean fiber areas in individuals with the wild-type CC genotype for rs1815739 (R577X) were significantly larger for type IIA fibers [1394.33 µm2 (572.77 µm2)] than in those with the TC and TT genotypes [832.61 µm2 (602.43 µm2) and 526.58 µm2 (432.21 µm2) (P = 0.014). Similar results for Q523R missense and intronic variants.

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# *ACTN3* genotype influences masseter muscle characteristics and self-reported bruxism

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## Abstract

**Objectives:** Main aim of the study was to explore the association between genetic polymorphisms in *ACTN3* and bruxism. Secondary objectives included masseter muscle phenotypes assessment between bruxers and non-bruxers and according to genetic polymorphisms in *ACTN3*.

**Materials and Methods:** Fifty-four patients undergoing orthognathic surgery for correction of their malocclusion were enrolled. Self-reported bruxism and temporomandibular disorders status were preoperatively recorded. Saliva samples were used for *ACTN3* genotyping. Masseter muscle samples were collected bilaterally at the time of orthognathic surgery to explore the muscle fiber characteristics.

**Results:** There were significant differences in genotypes for rs1815739 (R577X nonsense) ($p = 0.001$), rs1671064 (Q523R missense) ($p = 0.005$), and rs678397 (intronic variant) ($p = 0.001$) between bruxers and non-bruxers. Patients with self-reported bruxism presented a larger mean fiber area for types IIA ($p = 0.035$). The mean fiber areas in individuals with the wild-type CC genotype for rs1815739 (R577X) were significantly larger for type IIA fibers (1394.33 $\mu m^2$

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AUTHOR CONTRIBUTIONS

**Romain Nicot:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Validation; Visualization; Writing-original draft. **Gwénaël Raoul:** Supervision; Validation; Writing-review & editing. **Alexandre Vieira:** Data curation; Formal analysis; Validation; Writing-review & editing. **Joël Ferri:** Supervision; Validation; Writing-review & editing. **James J. Sciote:** Data curation; Formal analysis; Funding acquisition; Investigation; Project administration; Resources; Software; Supervision; Validation; Writing-review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVALS FOR THE STUDY

Temple University IRB: Certificate 13438. University of Lille CPP (Committee for Personal Protection): CPP12/44. University of Pittsburgh IRB: Certificate PRO12080373.

PATIENT CONSENT STATEMENT

Subjects were enrolled for study after participation was discussed and an informed consent was signed.

PEER REVIEW

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[572.77 $\mu m^2$]) than in those with the TC and TT genotypes (832.61 $\mu m^2$ [602.43 $\mu m^2$] and 526.58 $\mu m^2$ [432.21 $\mu m^2$] [$p = 0.014$]). Similar results for Q523R missense and intronic variants.

**Conclusions:** ACTN3 genotypes influence self-reported bruxism in patients with dentofacial deformity through specific masseter muscle fiber characteristics.

**Keywords**

ACTN3 protein; human; bruxism; malocclusion; masseter muscle; sleep bruxism; temporomandibular joint disorders

## 1 | INTRODUCTION

Dentofacial deformities often develop as a complex trait condition that is influenced by a combination of genes acting on bone, teeth, and skeletal muscle (Sciote et al., 2013). Indeed, previous studies have shown that the masticatory muscle genotype and phenotype contribute greatly to variations in the vertical dimension of facial growth (Ringqvist, 1974; Rowlerson et al., 2005; Sciote et al., 2012) or mandibular asymmetry (Raoul et al., 2011), through changes in the size and proportion of masseter muscle fibers. One gene of interest which is well documented to influence muscle performance and the proportion of fiber types is *ACTN3* (Vincent et al., 2007). It encodes alpha-actinin-3, which is a Z-disc structural protein found only in type II muscle fibers (North & Beggs, 1996), functions to enhance the force, speed, and strength of skeletal muscle contraction. A common nonsense mutation, R577X, was identified in the *ACTN3* gene and results in a lack of protein expression due to the production of a stop codon at residue 577 (North et al., 1999). The absence of alpha-actinin-3 protein due to this polymorphism has been shown to change fiber type proportion, skeletal muscle metabolism, bone mineral density, and is associated with class II and deep-bite skeletal malocclusions (Zebrick et al., 2014). This mutated genotype also results in significantly smaller diameters of fast type II fibers in masseter muscles. Patients with facial asymmetry consisting of a longer ramus but dimensionally smaller condyles are more likely to have self-reported temporomandibular disorder (TMD) symptoms and significantly more common clinical diagnosis of TMD, with masticatory myalgia most prominent (Nicot et al., 2020). Genotyping revealed two significant associations of these conditions with the *ACTN3* polymorphisms rs1671064 (Q523R missense) and rs678397 (intronic single nucleotide polymorphism [SNP]) and one allele rs1815739 (R577X nonsense) (Nicot et al., 2020). In addition, the rs678397, rs1671064, and rs1815739 polymorphisms in *ACTN3* have been reported to be associated with bruxism in children (Calvano Küchler et al., 2020).

Bruxism is a complex oral condition leading to a number of clinical problems, including orofacial pain, tooth wear, and failure of dental restorative treatments. According to international expert consensus, bruxism was defined as (Lobbezoo et al., 2013) "*a repetitive jaw muscle activity characterized by clenching or grinding of the teeth and/or by bracing or thrusting of the mandible, having two distinct circadian manifestations. It can occur during sleep (indicated as sleep bruxism) or during wakefulness (indicated as awake bruxism).*" Since 2018, experts have defined awake bruxism as "*a masticatory muscle activity during wakeful-ness that is characterized by repetitive or sustained tooth contact and/or by bracing or thrusting of the mandible and is not a movement disorder in otherwise*

*healthy individuals*," while sleep bruxism is defined as "*a masticatory muscle activity during sleep that is characterized as rhythmic (phasic) or nonrhythmic (tonic) and is not a movement disorder or a sleep disorder in otherwise healthy individuals*."(Lobbezoo et al., 2018; Manfredini et al., 2019, 2021) The prevalence of sleep bruxism is reported to be about 16% in the adult population, while the prevalence of awake bruxism is 24% (Lobbezoo et al., 2012). Both types occur due to the contraction of jaw muscles. Awake bruxism occurs mainly in the form of tooth clenching or mandible bracing. This represents prolonged isometric contractions of the masticatory muscles that differ from motor activity with respect to the phasic, isotonic, and the sudden-onset contraction of rhythmic masticatory muscle activity that occurs in sleep bruxism episodes (Manfredini et al., 2021). Currently, there is a paradigm shift in relation to bruxism from a pathology to a behavior, albeit with some clinical links to TMD (Manfredini et al., 2021), and a genetic component (Calvano Küchler et al., 2020; Vieira et al., 2020) Nevertheless, its place among the onset of pain conditions is particularly complex and varies according to age. A recent meta-analysis (de Oliveira Reis et al., 2019) focusing on the pediatric population showed that children with bruxism have a greater chance of developing TMD (de Oliveira Reis et al., 2019). Conversely, evidence provided in the systematic review of the adult population (Jiménez–Silva et al., 2017) was inconclusive, but suggested that bruxism is associated with TMD (Jiménez-Silva et al., 2017). Similarly, the systematic review by Baad–Hansen et al., in 2019 did not support a direct linear causal relationship between bruxism and musculoskeletal pain symptoms, pointing more in the direction of a multifaceted relationship dependent on the presence of other risk factors (Baad-Hansen et al., 2019). On the other hand, bruxism and dysfunctional oral habits were shown to be risk factors for the presence of TMD symptoms after combined orthodontic and surgical treatment in a population with dentofacial deformities (Bruguiere et al., 2019).

As *ACTN3* genotypes have been associated with bone growth-skeletal malocclusion characteristics and muscle fiber types—masticatory functional differences in patients undergoing surgical treatment for dentofacial deformity, we hypothesized that variations in these genotypes could contribute to bruxism through specific patterns of motor unit recruitment and fiber type. Therefore, the main aim of this study was to explore the association between genetic polymorphisms in *ACTN3* and bruxism in a cohort of young adults with dentofacial deformities. In addition, we investigated the masseter muscle histomorphometry between bruxers and non-bruxers. Finally, as alpha-actinin-3 loss has been shown to be associated with changes in muscle fiber characteristics, we evaluated the association between genotypes in ACTN3 variants altering the protein structure and masseter muscle histomorphometry to determine how the relationships between genetics and physiology might underly bruxism in adults. Given the potential role of bruxism in the onset of TMD, we extended these investigations to TMDs.

## 2 | MATERIAL AND METHODS

### 2.1 | Participants

Patients undergoing orthognathic surgery for correction of malocclusion were recruited from the University of Lille Department of Oral and Maxillofacial Surgery (France). All

patients with a neurological or systemic condition, with developmental disorders of the temporomandibular joint (TMJ) that might influence TMD, or with medication influencing bruxism development, were excluded from the study.

All procedures performed in the study were in accordance with the ethical standards of the Helsinki declaration. All participants provided written informed consent, and the research protocol was validated by a French Independent Ethics Committee (CPP12/44), the Temple University (Certificate 13438), and the University of Pittsburgh (Certificate PRO12080373) IRB Committees.

Age, sex, and bruxism status, as well as TMJ symptoms, were recorded during preoperative examination. Cephalometric analysis was performed to determine the craniofacial morphologic diagnosis. Saliva samples collected from all subjects were stored in Oragene® kits (DNA Genotek) and used for DNA extraction and posterior genotyping. Masseter muscle samples were collected bilaterally from the area of the deep anterior masseter muscle, by using an intraoral approach at the time of orthognathic surgery. Indeed, all included patients had a bilateral sagittal split osteotomy by Epker surgical procedure, which permits to easily perform a masseter biopsy without additional surgical incision. Masseter muscle samples were then snap-frozen in isopentane cooled with liquid nitrogen to ensure optimal preservation and avoid any disintegration of any tissue parts, and stored at −80°C prior to histologic evaluation and gene expression analysis.

### 2.2 | Bruxism status and TMD status

Bruxism status was recorded during the preoperative interview and clinical examination. According to international expert consensus (Lobbezoo et al., 2013, 2018), bruxism was diagnosed based on non-instrumental approaches (self-reporting and clinical examination). Each patient was asked whether he/she engaged in grinding or clenching his/her teeth, and in bracing or thrusting of the mandible. Bruxism dental signs were clinically collected (attrition; study wear facets; abfractions; chipping, breaking, cracks and fractures; periodontal recession and bone loss; tooth mobility; etc.)

According to the diagnostic criteria for TMDs (DC/TMD) (Dworkin & LeResche, 1992; Reiter et al., 2012; Schiffman et al., 2014), TMD status was classified into five types of symptoms: myalgia, arthralgia, headache attributed to TMD, disc displacement with reduction, and disc displacement without reduction. As defined in the DC/TMD, myalgia was diagnosed based on history and clinical examination as a pain of muscular origin that is affected by jaw movement, function, or parafunction; replication of this pain occurs with provocation testing of masticatory muscles.

### 2.3 | Cephalometric analysis

Cephalometric data were assessed and classified according to Delaire analysis (Delaire et al., 1981). This allowed us to precisely determine the exact craniofacial morphotype of each patient. Patients were then classified into one of six craniofacial morphologic groups that included a variation of sagittal skeletal jaw malocclusion (Class II or Class III), and a variation of vertical skeletal occlusal relationship (open bite, deep bite, or normal bite).

Anterior facial asymmetry was identified by measuring the deviation of the mandibular dental midline, and asymmetry was defined as a deviation ≥2 mm.

### 2.4 | Genotyping

Three SNPs in *ACTN3* (rs1815739 [R577X nonsense], rs1671064 [Q523R missense], and rs678397 [intronic variant]) were selected for genotyping of all subjects. To determine if specific allelic variants were overrepresented in patients with bruxism, TMD subtypes, and muscle fiber types, we analyzed these SNPs using TaqMan chemistry and end-point analysis in an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems), as described previously (Zucchero et al., 2004).

### 2.5 | Analysis of masseter muscles

Serial sections (10 $\mu$m thickness) were prepared from frozen muscles. The sections were mounted on glass microscope slides for immunostaining with antibodies specific for myosin heavy chain (MyHC) isoforms as described previously (Sciote et al., 1994). The antibodies used were as follows: Anti-I (BA-F8), Anti-fast (BF-35), Anti-IIA (SC-71), Anti-neonatal, and Anti-atrial. We then classified masseter fibers into the following six groups: type I, type hybrid (containing both type I and II MyHC), type IIA, type IIX, neonatal type, and atrial type (Sciote et al., 1994). Neonatal and atrial types both contained the neonatal and/or α–cardiac MyHC in combination with other type I and II isoforms (Korfage et al., 2005a). Type I fibers are slow-contracting and fatigue-resistant, and function most commonly to produce the jaw posture freeway space that maintains the airway. Type II fibers are fast contracting and are either fatigue-resistant or fatigable. Hybrid type fibers, which are a very unusual and distinctive group, are found in masseter muscles, which combine slow and fast contractile properties. Hybrid fibers can be found in certain states of skeletal muscle pathology, but are almost never present in normal limb muscle. For fiber type classification, only tissue section series with consistent antibody reactions for all stains and acceptable morphology of muscle fibers that were clear in transverse section were used. All fibers within the selected areas were type-classified and their cross-sectional areas were analyzed with Image Image J software. Tests for measurement error included intra-rater reliability in determination of fiber area (by repeating morphometric tracing of all fiber areas in one biopsy by one examiner), which resulted in an $R^2$ value of 0.94. Listed histomorphometric data included fiber type, the number of each fiber, their percentage of occupancy, and their mean surface area.

### 2.6 | Statistical analysis

Patient characteristics were analyzed using the usual rules for descriptive statistics: frequencies and percentages for categorical variables, and mean and standard deviation for quantitative variables. After ensuring compliance with Hardy–Weinberg equilibrium, allelic frequencies between bruxers and non-bruxers were compared using the $\chi^2$ test or Fisher's exact test in a dominant model. One-way analysis of variance (ANOVA) was used for comparisons of the phenotypic characteristics of participants (bruxism status, TMD subtypes, or muscle phenotypes) in various *ACTN3* genotypes. The Bonferroni correction method was used to correct the $p$-values in multiple testing, with a $p < 0.017$ ($p = 0.05/3$, where 3 is the number of SNPs included in this study) considered to indicate statistical

significance. To evaluate the effect of *ACTN3* genotypes on phenotype, multinomial logistic regression was used to adjust for the association of these SNPs with age, sex and biometrics, and the related odds ratio (OR) values were determined. Muscle phenotype quantitative variables between bruxers and non-bruxers were analyzed using Student's *t*-test. When the distribution of the variable was not normal, the nonparametric Wilcoxon test was performed. All statistical analysis was performed using SPSS software (SAS Institute), and *p values* < 0.05 were considered to indicate statistical significance.

## 3 | RESULTS

We recruited 54 orthognathic surgery patients who were systemically healthy and without genetic craniofacial syndromes, other growth disturbances, or reported trauma. This sample included a larger proportion of women 39 (72.22%), young people (18 years [16; 29.75]), and Class II dentofacial deformity patients (74.07%). Details of the age, sex, and biometric characteristics of the study population are listed in Table 1. There was no significant difference between the two groups (bruxers and non-bruxers) regarding age, sex, or biometric characteristics of patients, suggesting that the two populations were similar. Regarding TMD diagnoses, there was no difference in the proportion of myalgia ($p = 0.67$) or of disc displacement with reduction ($p = 0.34$) using Fisher's exact between bruxers and non-bruxers.

### 3.1 | Association between bruxism and ACTN3 genotypes or alleles

There were significant differences in genotypes for SNPs rs1815739 (R577X nonsense) ($p = 0.001$), rs1671064 (Q523R missense) ($p = 0.005$), and rs678397 (intronic variant) ($p = 0.001$) between bruxers and non-bruxers. There were also significant differences in alleles for rs1815739 (R577X nonsense) ($p < 0.002$) rs1671064 (Q523R missense) ($p = 0.013$), and rs678397 (intronic variant) ($p = 0.000$) between the two groups (Table 2).

### 3.2 | Association between masseter fiber types and bruxers

Patients with self-reported bruxism presented a larger mean fiber area for types IIA, atrial, and neonatal. The mean area of type IIA fibers was 1227.29 $\mu m^2$ (508.27 $\mu m^2$) for bruxers and 774.33 $\mu m^2$ (614.24 $\mu m^2$) for non-bruxers ($p = 0.035$), while the mean fiber area of the atrial type was 564.67 $\mu m^2$ (517.04 $\mu m^2$) for bruxers and 258.33 $\mu m^2$ (406.50 $\mu m^2$) for non-bruxers ($p = 0.046$) and the mean fiber area of the neonatal type was 74838.51 $\mu m^2$ (235827.73 $\mu m^2$) for bruxers and 246.52 $\mu m^2$ (466.19 $\mu m^2$) for non-bruxers ($p = 0.035$) (Table 3).

There was also a significant difference in the percent occupancy for the atrial type fibers, with a larger percent occupancy in the bruxer group than in the non-bruxer group (8.02% [14.33%] vs. 2.52% [5.03%]; [$p = 0.042$]). For neonatal type fibers, there was a tendency for a larger percent occupancy in the bruxer group than in the non-bruxer group (9.77% [29.47%] vs. 1.86% [3.75%]), although this difference did not reach the level of statistical significance ($p = 0.085$). Finally, bruxers had significantly more atrial fibers than non-bruxers (14.15 [27.46] vs. 3.24 [5.74]; $p = 0.016$).

Figure 1 shows four low power (10×) immunohistochemical staining images of fast and slow myosin in masseter fibers of a patient with homozygous mutated *ACTN3* genotypes for rs1815739 (R577X nonsense), rs1671064 (Q523R missense), and rs678397 (intronic variant), compared with the fibers of a patient with the wild-type genotypes for all three positions. Differences in overall fast versus slow myosin-fiber composition of masseter fibers are highlighted depending on the *ACTN3* genotype.

### 3.3 | Association between ACTN3 genotypes or alleles and masseter fiber type characteristics

For rs1815739 (R577X nonsense leading to absence of alpha-actinin-3), there were significant differences in the mean fiber areas among the genotypes for type IIA ($p = 0.014$) and neonatal type ($p < 0.0001$) and alleles for types I ($p = 0.014$) and IIA ($p = 0.003$). The mean fiber areas in individuals with the wild-type CC genotype were significantly larger for type IIA fibers (1394.33 $\mu m^2$ [572.77 $\mu m^2$]) than in those with the TC and TT genotypes (832.61 $\mu m^2$ [602.43 $\mu m^2$] and 526.58 $\mu m^2$ [432.21 $\mu m^2$], respectively). There were also significant differences in the percent occupancy among the genotypes for type I ($p < 0.011$) and neonatal type ($p < 0.0001$) fibers. Finally, there were significantly fewer type I/II hybrid fibers in patients with genotype CC than in patients with either of the other genotypes ($p = 0.009$) (Tables 4–6).

Similarly, for rs1671064 (Q523R missense), there were significant differences in mean fiber area in genotypes for type IIA ($p = 0.014$) and neonatal type ($p < 0.0001$) fibers and alleles for type I ($p = 0.032$) and IIA ($p = 0.014$) fibers. The mean fiber area for type IIA fibers of individuals with the wild-type AA genotype (1344.86 $\mu m^2$ [591.49 $\mu m^2$]) was significantly larger than that of individuals with the GA and GG genotypes (860.75 $\mu m^2$ [615.50 $\mu m^2$] and 526.58 $\mu m^2$ [432.21 $\mu m^2$], respectively). There were also significant differences in the percent occupancy among genotypes for type I ($p = 0.011$) and neonatal type ($p < 0.0001$) fibers. Type I/II hybrid fibers were also significantly fewer in individuals with the AA genotype than in those with either of the other genotypes ($p = 0.009$).

Finally, for rs678397 (intronic SNP), there were significant differences in the mean fiber area among the genotypes for type IIA ($p = 0.014$) and neonatal type ($p < 0.0001$) fibers and alleles for type I ($p = 0.004$), IIA ($p = 0.000$) and neonatal type ($p = 0.043$) fibers. The mean fiber area of type IIA fibers was significantly larger in participants with the wild-type CC genotype (1369.62 $\mu m^2$ [545.59 $\mu m^2$]) than that among individuals with the TC and TT genotypes (728.59 $\mu m^2$ [576.77 $\mu m^2$] and 547.11 $\mu m^2$ [385.48 $\mu m^2$], respectively). However, there were also significant differences in the percent occupancy of type I and neonatal type fibers ($p = 0.011$ and $p < 0.0001$, respectively). Similarly, type I/II hybrid fibers were also significantly fewer in participants with the CC genotype than in those with either of the other genotypes ($p = 0.009$).

Using multinomial logistic regression, we showed that carriers of TC/TT genotypes of *ACTN3 R577X* were at significantly lower risk of bruxism (OR = 0.128, 95%CI = 0.017–0.945; $p = 0.044$ and OR = 0.026, 95%CI = 0.001–0.531; $p = 0.018$, respectively) compared to individuals with the CC genotype. Carriers of the GG genotype of *ACTN3 Q523R* were at significantly lower risk of bruxism (OR = 0.025, 95%CI = 0.001–0.617; $p = 0.024$)

compared to individuals with the AA genotype. Finally, carriers of the TC/TT genotypes of *ACTN3 intronic variant* were also at significantly lower risk of bruxism (OR = 0.087, 95%CI = 0.011–0.718; $p = 0.023$ and OR = 0.046, 95%CI = 0.004–0.547; $p = 0.015$, respectively) compared to individuals with the CC genotype. Multinomial logistic regression showed no relevant associations regarding the muscle phenotypes (data not shown).

### 3.4 | Association between ACTN3 genotypes or alleles, masseter fiber types, and subtypes of TMD

No associations were identified among the different TMD subtypes, *ACTN3* genotypes, and the different phenotypic characteristics of the muscle (data not detailed).

In particular, there was no association between myalgia and genotype/alleles in alpha-actinin-3 altering variants rs1815739 (R577X nonsense) ($p = 0.143/p = 1$) and rs1671064 (Q523R missense) ($p = 0.168/p = 1$). There was no significant difference in the mean fiber area for all fiber types (all *p values* >0.2 except for neonatal type [$p = 0.135$]), in the percent occupancy for all fiber types (all *p values* >0.2 except for neonatal type [$p = 0.103$]), and in the number of each fiber (all *p values* >0.2 except for neonatal type [$p = 0.132$]).

In addition, there was no association between disc displacement with reduction and genotype/alleles in alpha-actinin-3 altering variants rs1815739 (R577X nonsense) ($p = 0.143/p = 1$) and rs1671064 (Q523R missense) ($p = 0.168/p = 1$). There was no significant difference in the mean fiber area for all fiber types (all *p values* >0.2 except for neonatal type [$p = 0.135$]), in the percent occupancy for all fiber types (all *p values* >0.2 except for type I [$p = 0.145$] and neonatal [$p = 0.103$]), and in the number of each fiber (all *p values* >0.2 except for neonatal types [$p = 0.132$]).

## 4 | DISCUSSION

Alpha-actinin-3, which is a cytoskeletal protein encoded by the *ACTN3* gene, binds actin filaments in skeletal muscle (North et al., 1999). In contrast to alpha-actinin-2, alpha-actinin-3 protein is present only in type II fast contracting fiber types, where they cross-link actin filaments with dense bodies located in the Z-disk of the sarcomere. This interaction helps to order the myofibril array, which is important in coordinating sarcomere contraction (Vincent et al., 2007). The *ACTN3 R577X* polymorphism (rs1815739) consists in a cytosine to thymine mutation at nucleotide 1586 in exon 16, which converts the arginine at position 577 to a stop codon, and produces three genotypes: CC (normal), CT (heterozygote), and TT (no alpha-actinin-3 protein) (North et al., 1999). Approximately 18% of the European population is homozygous for this common nonsense mutation (Yang et al., 2003).

*ACTN3* genotypes have been widely studied in human elite athletic population, mainly through *ACTN3 R577X* variations, since they represent a natural experimental model of a nonsense mutation contributing to muscle performance (Ma et al., 2013; Tharabenjasin et al., 2019). Compared with X allele carriers, studies have indicated that the RR genotype and R allele carriers have greater muscle size and strength (Broos et al., 2015; Kikuchi & Nakazato, 2015; Walsh et al., 2008), faster sprint times (Moran et al., 2007), and a higher proportion of fast-twitch muscle fibers (Ahmetov et al., 2011; Vincent et al., 2007).

Reciprocally, elite power sports athletes have been reported to have a higher frequency of the RR +RX genotype in their fast-twitch skeletal muscle compared to controls (Yang et al., 2003). Nevertheless, a recent meta-analysis by Tharabenjasin et al., in 2019 showed a significant association of the R allele with female elite power sports athletes, while no such association was identified for their male counterparts (Tharabenjasin et al., 2019). In a histomorphometric study focusing on the *vastus lateralis* muscle in young adult males, individuals carrying the RR genotype had type IIX, fast contracting-fatigable fibers, which were significantly larger in size and greater in number compared to individuals carrying the mutated XX genotype (Vincent et al., 2007). In this study, alpha-actinin-3 protein content was systematically higher in type IIX compared with type IIA fibers. Therefore, we propose that the *ACTN3* variant could be one of the genes that contributes to the heritability of fiber type distribution through its interaction with calcineurin. Alpha-actinin-3 also binds to calsarcin family signaling proteins located at the Z-disc (Frey & Olson, 2002). These, in turn, bind to the signaling protein calcineurin, which has a key role in determining fiber type and size through activation fiber type-specific gene expression pathways (Swoap et al., 2000). Calcineurin activity is increased when alpha-actinin-3 expression is lost, explaining the slower metabolic, physiological, and functional phenotypes associated with alpha-actinin-3 deficiency.

Increased calcineurin activity is associated with increased muscle plasticity, as demonstrated by an enhanced adaptive response to endurance training in *ACTN3* knockout mice and an increased switch in muscle fiber type from fast-twitch glycolytic fibers (type IIX) toward fast-twitch oxidative fibers (type IIA) (Seto et al., 2013). Myofibers are known to undergo a progressive transition due to changes in muscle activity, usually changing from type IIX to type IIA to type I when muscles are loaded (Yamada et al., 2020). Variation in the pellet hardness of a rat diet is an experimental model of this masseter fiber type transition. Studies have shown a reduction in fiber volume (Kawai et al., 2010; Kiliaridis et al., 1988; Kiliaridis & Shyu, 1988; Miehe et al., 1999) and a significant increase in type IIB fibers in the deep masseter fibers after consuming soft food compared to those on a hard food diet (Saito et al., 2002). Based on this model, Saito et al. suggested that similar changes that might be induced by a soft diet in human masseter muscle could result in a phenotypic transition from type I/IIA fibers, which are predominant under normal conditions, to type IID/X or even to IIB' (Saito et al., 2002). More recently, Takasu et al. reported evidence in support of previous results in a rabbit model, showing that a liquid diet caused a reduction in the diameter of fast-twitch muscle fibers and increased the proportion of fast-twitch fibers (Takasu et al., 2019).

In our study, we showed a significant association of participants with self-reported bruxism with wild-type genotypes and alleles for SNPs rs1671064 (Q523R missense), rs1815739 (R577X nonsense), and rs678397 (intronic SNP). In addition, these participants had a larger mean fiber area for type IIA and an increased number of atrial/neonatal type fibers. Type I fibers are characterized by low force, power, and speed production and high endurance, while type IIX fibers are characterized by high force, power, and speed production and low endurance, with type IIA with intermediate characteristics (Korfage et al., 2005a). Therefore, our results are consistent with previous reports and the relevant experimental models showing a specific pattern of muscle phenotype characterized by larger fast-twitch

oxidative fibers and fewer hybrid fibers that confer intermediate contraction velocity and fatiguability on masseter muscle.

Despite bruxism was not a peripheral condition, these characteristics seem to provide favorable pathophysiological conditions that lead to its clinical expression. Moreover, compared with non-bruxers, bruxers had more atrial/neonatal type fibers, with higher percent occupancy. This particular fiber type has been shown to be commonly co-expressed with either type I or type IIA fibers, indicating that α–cardiac MyHC, which forms atrial fibers, is an intermediate isoform between the slow MyHC-I and the fast MyHC-IIA isoforms (Hämäläinen & Pette, 1997; Peuker et al., 1998). In addition, it has been speculated that fibers with different combinations of myosin composition increase the capacity of the jaw muscles to perform a wide variety of motor tasks, since these fibers have contractile properties that lie between those of pure fibers (Korfage et al., 2005b). The greater the composition of fibers, the more a continuum exists in contractile properties that could contribute to a precise modulation of mandibular position and force. In addition, all three common genotypes were associated with larger mean areas of type IIA fibers, although we did not find any difference in mean area of the type IIX fibers, indicating a tripartite relationship between bruxism, *ACTN3* genotypes, and type IIA fiber enlargement. Both wild-type genotypes of rs1671064 (Q523R missense) and rs1815739 (R577X nonsense) also showed lower type I/II hybrid mean fiber area, while this parameter was increased for rs678397 (intronic SNP), suggesting a role of alpha-actinin-3 in bruxism, although the proportion of hybrid fibers changes when alpha-actinin-3 expression is lost. Moreover, multinomial logistic regression showed that the mutant *ACTN3* genotypes protect against bruxism. Therefore, although muscle phenotype is strongly influenced by genetics, it is also subject to environmental influences (Isola et al., 2018). It is probable that, to some extent, the histomorphometric results are linked to fiber type transitions due to masseter muscle training or jaw disproportion, thus distorting the results of the statistical analysis (Nicot et al., 2020; Zebrick et al., 2014). This could explain why enlargement of type IIA fibers was not found to be a risk factor in the multinomial logistic regression.

On the other hand, this research supports the current paradigm switch, in which bruxism is considered to be an oral behavioral more than a pathology in most cases. Indeed, no association was found between bruxism and the different subtypes of TMD. Nevertheless, these results should be interpreted with caution given the small sample size. Moreover, all our results suggest the high adaptability of muscle pattern based on the possibility of fiber transition.

Our results are also particularly interesting from the perspective of the pathophysiology of botulinum toxin injections into masseter and temporal muscles for the treatment of bruxism. This approach produces a reduction in the muscular force and frequency of bruxism events and is therefore more and more used in the management of bruxism to improve the quality of life of patients (Ågren et al., 2020; Almukhtar & Fabi, 2019; Fernández-Núñez et al., 2019; Sendra et al., 2020; Villa et al., 2019). Indeed, injected masseters show a steep increase in the size of type IIX fibers, whereas fast fibers decreased by approximately 50% (Korfage et al., 2012). Therefore, botulinum toxin injections lead to a fiber type transition that could be unfavorable for the development of bruxism. These results are in accordance

with those reported by Tsai et al., in which a reduction in muscle fiber size and transition of muscle fiber subtypes from type IIA to IIX or IIB was found to occur due to reduced masticatory function in a rat model treated with botulinum toxin injection (Gedrange et al., 2013; Tsai et al., 2012). Fiber transition after masticatory muscle botulinum toxin injections in humans remains to be investigated.

The initial objective of study was to investigate the musculoskeletal heritable influences on malocclusions. Therefore, the main limitation of this study concerns the diagnosis of bruxism, which was based on self-reporting and did not differentiate between the two patterns of the condition. Nevertheless, this study is, to date, the first to explore polymorphisms in the genes of interest and the histomorphometric patterns in the related muscles in human self-reported bruxism in patients with dentofacial deformity.

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## DATA AVAILABILITY STATEMENT

All relevant data are within the paper and its Supporting Information files.

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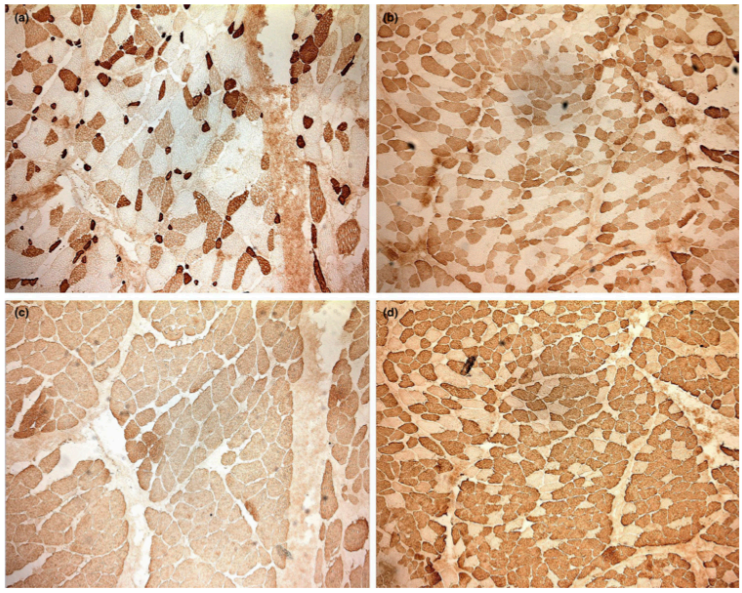


**FIGURE 1.**

Low power (10×) immunohistochemical staining images of fast (a) and slow (c) myosin in masseter fibers of a patient with homozygous mutated ACTN3 genotypes for rs1815739 (R577X nonsense), rs1671064 (Q523R missense), and rs678397 (intronic variant), compared with the fast (b) and slow (d) myosin in fibers of a patient with the wild-type genotypes for all three positions

**TABLE 1**

Description of the study population

| **Characteristics of the study population** | ***n* = 54** |
|---|---|
| Age md (Q1;Q3) | 18 (16;29.75) |
| Females *n* (%) | 39 (72.22) |
| Biometrical characteristics: | |
| Sagittal *n* (%) | |
| Class I | 1 (1.85) |
| Class II | 40 (74.07) |
| Class III | 13 (24.07) |
| Vertical *n* (%) | |
| Normal bite | 19 (35.18) |
| Open bite | 25 (46.30) |
| Deep bite | 10 (18.52) |
| Mandibular asymmetry *n* (%) | |
| No asymmetry | 33 (61,11) |
| Mandibular asymmetry >2 mm | 21 (38,89) |
| Bruxism *n* (%) | 10 (18.52) |
| Myalgia *n* (%) | 9 (16.67) |
| Arthralgia *n* (%) | 4 (7.41) |
| Headache attributed to TM D *n* (%) | 2 (3.70) |
| Disc displacement with reduction *n* (%) | 9 (16.67) |
| Disc displacement without reduction *n* (%) | 1 (1.85) |

Abbreviations: md, median; n (%), number of observation (percentage); *n*, number of observations; Q1;Q3, interquartile.

**TABLE 2**

Comparison of allelic and genotypic frequencies between bruxers and non-bruxers for rs1815739 (R577X nonsense), rs1671064 (Q523R missense), and rs678397 (intronic variant)

| | rs1815739 (R577X) | | | *p*-value | rs1671064 (Q523R) | | | *p*-value | rs678397 (Intronic) | | | *p*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CC | TC | TT | genotype/allele | AA | GA | GG | genotype/allele | CC | TC | TT | genotype/allele |
| Bruxers | 5 (50.0) | 5 (50.0) | 0 (0) | $p = 0.001/0.002$ | 4 (40.0) | 6 (60.0) | 0 (0) | $p = 0.005/0.013$ | 6 (66.7) | 2 (22.2) | 1 (11.1) | $p = 0.001/0.000$ |
| Non-bruxers | 4 (9.1) | 28 (63.6) | 12 (27.3) | | 4 (9.1) | 28 (63.6) | 12 (27.3) | | 4 (9.8) | 23 (56.1) | 14 (34.1) | |

**TABLE 3**

Association between masseter fiber types and bruxer status

| | Bruxers ($n$ = 10) | Non-bruxers ($n$ = 44) | $p$ |
|---|---|---|---|
| Muscle fiber mean area ($\mu m^2$) | | | |
| Type I | 2010.24 (640.52) | 1695.14 (881.33) | 0.292 |
| Type hybrid | 1171.52 (487.33) | 1265.23 (735.93) | 0.704 |
| Type IIA | 1227.29 (508.27) | 774.33 (614.24) | **0.035** |
| Type IIX | 195.13 (617.07) | 489.37 (955.30) | 0.358 |
| Atrial | 564.67 (517.04) | 258.33 (406.50) | **0.046** |
| Neonatal | 74838.51 (235827.73) | 246.52 (466.19) | **0.035** |
| Muscle fiber percent occupancy (%) | | | |
| Type I | 38.72 (19.46) | 43.46 (15.18) | 0.401 |
| Type hybrid | 19.47 (13.14) | 27.24 (17.80) | 0.199 |
| Type IIA | 23.66 (22.13) | 17.59 (12.99) | 0.253 |
| Type IIX | 0.36 (1.13) | 7.33 (16.16) | 0.181 |
| Atrial | 8.02 (14.33) | 2.52 (5.03) | **0.042** |
| Neonatal | 9.77 (29.47) | 1.86 (3.75) | 0.085 |
| Number of fibers | | | |
| Type I | 42.55 (20.93) | 41.17 (26.18) | 0.877 |
| Type hybrid | 34.85 (24.82) | 30.09 (22.83) | 0.561 |
| Type IIA | 33.85 (20.20) | 35.67 (38.11) | 0.885 |
| Type IIX | 0.70 (2.21) | 7.65 (16.36) | 0.189 |
| Atrial | 14.15 (27.46) | 3.24 (5.74) | **0.016** |
| Neonatal | 1.40 (2.96) | 2.97 (6.27) | 0.447 |

Abbreviation: N, number of observation. All variables are expressed as means (standard deviations).

Bold values are statistically significant $p$-values.

**TABLE 4**

Muscle fiber mean area depending on *ACTN3* genotypes and alleles

| | | rs1815739 (R577X) | *p*-value Genotype/allele | | rs1671064 (Q523R) | *p*-value Genotype/allele | | rs678397 (intronic) | *p*-value Genotype/allele |
|---|---|---|---|---|---|---|---|---|---|
| Muscle fiber mean area in $\mu m^2$: my (SD) | | | | | | | | | |
| Type I | CC (N = 9) | 2374.72 (909.48) | 0.112/**0.014** | AA (8) | 2341.98 (966.59) | 0.112/**0.032** | CC (10) | 2412.88 (865.91) | 0.112/**0.004** |
| | TC (33) | 1591.90(671.20) | | GA (34) | 1622.62 (684.81) | | TC (25) | 1527.64 (647.01) | |
| | TT (12) | 1731.97 (1067.18) | | GG (12) | 1731.97 (1067.18) | | TT (15) | 1652.15 (966.67) | |
| Type I/II hybrid | CC (9) | 1337.94 (351.12) | 0.713/0.674 | AA (8) | 1341.21 (375.22) | 0.713/0.684 | CC (10) | 1340.74 (331.16) | 0.713/0.535 |
| | TC (33) | 1202.85 (688.74) | | GA (34) | 1206.05 (678.49) | | TC (25) | 1211.85 (609.99) | |
| | TT (12) | 1304.16(912.23) | | GG (12) | 1304.16(912.23) | | TT (15) | 1167.82 (881.97) | |
| Type IIA | CC (9) | 1394.23 (572.77) | **0.014/0.003** | AA (8) | 1344.86 (591.49) | **0.014/0.014** | CC (10) | 1369.62 (545.59) | **0.014/0.000** |
| | TC (33) | 832.61 (602.43) | | GA (34) | 860.75 (615.50) | | TC (25) | 728.59 (576.77) | |
| | TT (12) | 526.58 (432.21) | | GG (12) | 526.58 (432.21) | | TT (15) | 547.11 (385.48) | |
| Type IIX | CC (9) | 310.84 (675.85) | 0.312/0.657 | AA (8) | 105.78 (299.18) | 0.312/0.269 | CC (10) | 279.76 (644.74) | 0.312/0.544 |
| | TC (33) | 354.75 (661.34) | | GA (34) | 401.70 (706.47) | | TC (25) | 409.68 (701.42) | |
| | TT (12) | 748.16 (1483.10) | | GG (12) | 748.17 (1483.10) | | TT (15) | 598.53(1350.63) | |
| Atrial type | CC (9) | 437.67 (549.14) | 0.115/0.365 | AA (8) | 356.62 (526.36) | 0.115/0.775 | CC (10) | 479.14 (534.08) | 0.115/0.219 |
| | TC (33) | 346.75 (435.13) | | GA (34) | 368.50(446.85) | | TC (25) | 389.69 (446.48) | |
| | TT (12) | 135.95 (337.29) | | GG (12) | 135.95 (337.29) | | TT (15) | 108.76 (304.23) | |
| Neonatal type | CC (9) | 588.04 (670.96) | **<0.0001**/0.667 | AA (8) | 463.62 (596.05) | **<0.0001**/0.685 | CC (10) | 75130.67 (235725.34) | **<0.0001/0.043** |
| | TC (33) | 22777.42 (129834.71) | | GA (34) | 22151.15 (127904.03) | | TC (25) | 198.36 (366.05) | |
| | TT (12) | 198.66(557.65) | | GG (12) | 198.66(557.65) | | TT (15) | 197.76 (510.39) | |

Abbreviations: my, mean; SD, standard deviation.

Bold values are statistically significant *p*-values.

**TABLE 5**

Muscle fiber percent occupancy depending on *ACTN3* genotypes and alleles

| | | rs1815739 (R577X) | *p*-value genotype/allele | | rs1671064 (Q523R) | *p*-value genotype/allele | | rs678397 (intronic) | *p*-value genotype/allele |
|---|---|---|---|---|---|---|---|---|---|
| Muscle fiber percent occupancy in %: my (SD) | | | | | | | | | |
| Type I | CC (*N* = 9) | 47.49 (13.09) | 0.011/0.318 | AA (8) | 48.31 (13.74) | **0.011**/0.277 | CC (10) | 43.13 (18.51) | **0.011**/0.857 |
| | TC (33) | 38.93 (15.66) | | GA (34) | 38.99 (15.43) | | TC (15) | 37.83 (11.34) | |
| | TT (12) | 49.00 (16.85) | | GG (12) | 49.00 (16.84) | | TT (25) | 49.40 (15.52) | |
| Type I/II hybrid | CC (9) | 20.27 (10.61) | 0.199/0.294 | AA (8) | 20.13 (11.33) | 0.199/0.316 | CC (10) | 18.47 (11.52) | 0.199/0.085 |
| | TC (33) | 27.64 (19.16) | | GA (34) | 27.46 (18.90) | | TC (15) | 31.63 (17.73) | |
| | TT (12) | 24.92 (15.47) | | GG (12) | 24.92 (15.47) | | TT (25) | 24.14 (16.91) | |
| Type IIA | CC (9) | 25.18 (10.71) | 0.203/0.160 | AA (8) | 25.17 (11.45) | 0.203/0.191 | CC (10) | 22.69 (12.80) | 0.203/0.173 |
| | TC (33) | 18.49 (17.25) | | GA (34) | 18.69 (17.03) | | TC (15) | 16.47 (13.47) | |
| | TT (12) | 14.53 (9.22) | | GG (12) | 14.53 (9.22) | | TT (25) | 16.79 (10.87) | |
| Type IIX | CC (9) | 1.82 (4.29) | 0.877/0.353 | AA (8) | 1.60 (4.53) | 0.877/0.363 | CC (10) | 1.64 (4.08) | 0.877/0.322 |
| | TC (33) | 6.39 (14.91) | | GA (34) | 6.31 (14.69) | | TC (15) | 6.98 (15.69) | |
| | TT (12) | 8.27 (19.43) | | GG (12) | 8.27 (19.43) | | TT (25) | 6.2 (17.57) | |
| Atrial type | CC (9) | 3.94 (4.65) | 0.589/0.867 | AA (8) | 3.39 (4.68) | 0.589/0.952 | CC (10) | 3.55 (4.55) | 0.589/0.939 |
| | TC (33) | 4.30 (9.35) | | GA (34) | 4.42 (9.23) | | TC (15) | 5.48 (10.47) | |
| | TT (12) | 1.15 (3.35) | | GG (12) | 1.15 (3.35) | | TT (25) | 0.92 (3.01) | |
| Neonatal type | CC (9) | 1.30 (2.45) | **<0.0001**/0.611 | AA (8) | 1.14 (2.59) | **<0.0001**/0.654 | CC (10) | 10.53 (29.27) | **<0.0001**/0.071 |
| | TC (33) | 4.39 (16.55) | | GA (34) | 4.27 (16.30) | | TC (15) | 1.68 (3.24) | |
| | TT (12) | 2.13 (5.09) | | GG (12) | 2.13 (5.09) | | TT (25) | 2.13 (4.73) | |

Abbreviations: my, mean; SD, standard deviation.

Bold values are statistically significant *p*-values.

**TABLE 6**

Muscle fiber number depending on *ACTN3* genotypes and alleles

| | | rs1815739 (R577X) | *p*-value genotype/allele | | rs1671064 (Q523R) | | *p*-value genotype/allele | rs678397 (intronic) | *p*-value genotype/allele |
|---|---|---|---|---|---|---|---|---|---|
| Muscle fiber percent occupancy in %: my (SD) | | | | | | | | | |
| Type I | CC (*N* = 9) | 39.17 (21.80) | 0.708/0.771 | AA (8) | 36.63 (21.84) | 0.708/0.563 | CC (10) | 42.50 (23.10) | 0.708/0.738 |
| | TC (33) | 42.68 (23.21) | | GA (34) | 43.18 (23.04) | | TC (25) | 40.42 (18.77) | |
| | TT (12) | 39.67 (33.37) | | GG (12) | 39.67 (33.37) | | TT (15) | 38.63 (29.83) | |
| Type I/II hybrid | CC (9) | 27.00 (17.71) | **0.009**/0.576 | AA (8) | 22.56 (12.48) | **0.009**/0.267 | CC (10) | 32.90 (25.04) | **0.009**/0.940 |
| | TC (33) | 35.49 (26.47) | | GA (34) | 36.28 (26.47) | | TC (25) | 38.98 (25.27) | |
| | TT (12) | 21.54 (11.06) | | GG (12) | 21.54 (11.06) | | TT (15) | 21.10 (12.69) | |
| Type IIA | CC (9) | 33.94 (13.31) | 0.614/0.899 | AA (8) | 31.44 (11.73) | 0.614/0.739 | CC (10) | 31.85 (14.19) | 0.614/0.681 |
| | TC (33) | 30.05 (21.50) | | GA (34) | 30.75 (21.56) | | TC (25) | 29.78 (21.46) | |
| | TT (12) | 50.92 (64.74) | | GG (12) | 50.92 (64.74) | | TT (15) | 49.53 (58.30) | |
| Type IIX | CC (9) | 2.22 (4.66) | 0.869/0.370 | AA (8) | 1.63 (4.60) | 0.869/0.339 | CC (10) | 2.00 (4.45) | 0.869/0.327 |
| | TC (33) | 7.33 (17.11) | | GA (34) | 7.32 (16.85) | | TC (25) | 8.40 (18.65) | |
| | TT (12) | 6.79 (14.22) | | GG (12) | 6.79 (14.22) | | TT (15) | 5.43 (12.92) | |
| Atrial type | CC (9) | 6.06 (10.03) | 0.713/0.845 | AA (8) | 6.69 (10.53) | 0.713/0.743 | CC (10) | 5.75 (9.51) | 0.713/0.949 |
| | TC (33) | 6.61 (15.77) | | GA (34) | 6.44 (15.56) | | TC (25) | 8.24 (17.81) | |
| | TT (12) | 0.96 (3.32) | | GG (12) | 0.96 (3.32) | | TT (15) | 0.77 (2.97) | |
| Neonatal type | CC (9) | 1.50 (2.72) | 0.801/0.511 | AA (8) | 1.69 (2.84) | 0.801/0.607 | CC (10) | 2.10 (3.19) | 0.801/0.646 |
| | TC (33) | 3.39 (6.93) | | GA (34) | 3.29 (6.85) | | TC (25) | 3.90 (7.72) | |
| | TT (12) | 1.58 (3.72) | | GG (12) | 1.58 (3.72) | | TT (15) | 1.73 (3.65) | |

Abbreviations: my, mean; SD, standard deviation.

Bold values are statistically significant *p*-values.

# Partie V : Synthèse du travail et perspectives

## I. Rôle des génotypes d'ENPP1 et d'ESR1 sur la géométrie condylienne et sur la santé articulaire temporomandibulaire avant et après traitement chirurgical des dysmorphoses dentofaciales

Au cours du processus de minéralisation, l'hydroxyapatite est constituée par la cristallisation de calcium et de phosphate inorganique. L'ectonucleotide pyrophosphatase / phosphodiesterase 1 est une ecto-enzyme transmembranaire codée par le gène *ENPP1*, qui hydrolyse le pyrophosphate inorganique et inhibe la formation de l'hydroxyapatite. Cheung CL et al. (Cheung et al. 2009) ont mis en évidence par la cohorte *Framigham*, l'implication de certains polymorphismes nucléotidiques du gène *ENPP1* dans la densité minérale osseuse et la morpho-biométrie osseuse.

*Influence des génotypes d'ENPP1 et d'ESR1 sur le santé articulaire temporomandibulaire avant et après traitement chirurgical des dysmorphoses dentofaciales*

Il existait une association entre le polymorphisme nucléotidique rs858339 du gène *ENPP1* et la dysfonction temporomandibulaire préopératoire. Il existait proportionnellement plus de patients avec le génotype TT dans le groupe Score JPF préopératoire <6 (Absence de dysfonction temporomandibulaire préopératoire) par rapport au groupe Score JPF préopératoire ≥6 (Dysfonction temporomandibulaire préopératoire) et ceci de façon significative.

Il existait également une association statistiquement significative entre le polymorphisme nucléotidique rs1643821 du gène *ESR1* et l'aggravation symptomatique dysfonctionnelle des patients après chirurgie orthognathique.

*Génotypes d'ENPP1 et variation de la géométrie condylienne*

Le polymorphisme nucléotidique rs9373000 du gène *ENPP1* était associé au différentiel de hauteur de l'unité condylienne. Notamment, le génotype GG était identifié comme un facteur protecteur de la diminution de hauteur condylienne.

*Relation entre les génotypes d'ENPP1 et d'ESR1, les sous-classifications d'asymétrie craniofaciale et les dysfonctions temporomandibulaires avant et après traitement chirurgical des dysmorphoses dentofaciales*

La prévalence des dysfonctions temporomandibulaires était beaucoup plus élevée chez les patients présentant une asymétrie faciale. Le génotype rs6569759 du gène *ENPP1* était associé au groupe d'asymétrie 1, et le rs858339 était associé au groupe d'asymétrie 3. Le génotype rs858339 était également associé au déplacement discal réductible, à la myalgie des muscles masticateurs et à l'arthralgie. Le génotype SNP rs1643821 du gène *ESR1* était associé au groupe d'asymétrie 4. Les diagnostics de déplacement discal réductible, de myalgie des muscles masticateurs et d'arthralgie étaient très répandus dans les groupes d'asymétrie, et tous présentaient des associations statistiques fortes avec le polymorphisme rs858339 du gène *ENPP1*. Les patients du groupe 3 avaient les scores JPF préopératoires les plus élevés, et les groupes 2 et 3 étaient les plus susceptibles d'être guéris d'une dysfonction temporomandibulaire 1 an après le traitement.

**Au total :**

Ces résultats sont particulièrement intéressants comptes tenus du rôle morpho-fonctionnel de l'articulation temporomandibulaire. Une modification, même modérée de la morphologie du condyle mandibulaire peut troubler le fonctionnement correct de cette articulation. Le gène *ENPP1,* codant pour une ecto-enzyme transmembranaire fondamentale dans le processus de minéralisation, était associé aux dysfonctions temporomandibulaires, à certaines formes d'asymétrie faciale et à la morphologie condylienne. Plus précisément, le génotype TT du polymorphisme nucléotidique rs858339 du gène *ENPP1* était un facteur protecteur d'une dysfonction temporomandibulaire. Parallèlement, le génotype GG du polymorphisme nucléotidique rs9373000 a été identifié comme un facteur protecteur de la diminution de hauteur condylienne. Enfin, le génotype rs858339 était associé à la forme d'asymétrie atypique et était associé aux dysfonctions temporomandibulaires à type de déplacement discal réductible, de myalgie des muscles masticateurs et d'arthralgie. Toutefois, ces patients étaient les plus susceptibles d'être guéris de ces symptômes en postopératoire d'une chirurgie orthognathique. Ainsi, *ENPP1* constitue un gène d'intérêt dans la stabilité condylienne mandibulaire, semblant

être associé à la morphologie condylienne et à ses conséquences fonctionnelles. Il pourrait être un gène d'intérêt clé dans l'étude des facteurs génétiques associés aux résorptions condyliennes.

## II. Rôle des génotypes d'ACTN3 dans l'équilibre de l'appareil manducateur

Les alpha actinines sont des protéines d'ancrage des myofibrilles du muscle squelettique qui influencent les propriétés contractiles. *ACTN2* code pour l'alpha actinine 2 qui est exprimée dans les fibres lentes de type I et rapides de type II alors que *ACTN3* code pour l'alpha actinine 3, exprimée uniquement dans les fibres rapides. Le polymorphisme nucléotidique *ACTN3 R577X* est présent chez environ 18% des Européens, et résulte en une absence de protéine alpha actinine 3, conduisant à une diminution l'activité contractile rapide, une amélioration des performances d'endurance et une réduction de la masse osseuse et de la densité minérale osseuse.

*Etude de l'association entre les polymorphismes nucléotidiques d'ACTN3 et les principales dysmorphoses dentofaciales*

Il existait une association entre le génotype A*CTN3 577XX* et la présence d'une dysmorphose dento-squelettique de classe II et du caractère hypodivergent de la dysmorphose dentofaciale.
Ce génotype muté, résultant en l'absence de protéine alpha actinine 3 était associé à un diamètre significativement plus petit des fibres rapides de type II dans le muscle masséter. Toutefois, il semblait également résulter en une augmentation du pourcentage d'occupation des fibres de type II.

*Association entre ACTN3 et les gènes OPPERA liés à la douleur dans les dysmorphoses dentofaciales*

Il existait une association entre le génotype d'*ACTN3* et les dysmorphoses dentosquelettiques de classe II et de caractère *deep-bite*, le type de fibre musculaire du muscle masséter et une tendance à la significativité pour la présence d'une dysfonction temporomandibulaire, qui était souvent limité à des myalgies. Une expression différentielle dans le muscle masséter pour l'un des gènes *OPPERA* liés aux processus douloureux, *CACNA2D1* (sous-unité alpha-2 / delta-1

du canal calcique voltage-dépendant, actif dans la douleur neuropathique), a été retrouvé selon le sexe et entre les femmes avec et sans myalgie.

*Etude de l'association entre polymorphismes nucléotidiques d'ACTN3, stabilité condylienne et dysfonctions temporomandibulaires*

Près de la moitié des patients présentaient une asymétrie nécessitant une correction chirurgicale, qui pouvaient être subdivisée en quatre modèles morphologiques distincts. Les sujets avec un *ramus* plus long mais des condyles dimensionnellement plus petits étaient plus susceptibles d'avoir des symptômes de dysfonction temporomandibulaire auto-déclarés et un diagnostic clinique positif, principalement à type de myalgie.

Il existait une association très significative entre les allèles *R577X* et le remodelage condylien dans le groupe d'asymétrie faciale typique, l'allèle X (muté) étant plus élevé lorsque le modelage du condyle est normal.

*Etude de la relation entre les génotypes d'ACTN3, phénotype musculaire massétérin et bruxisme auto-déclaré, principale parafonction orale et facteur étiopathogénique des dysfonctions temporomandibulaires.*

Nous avons mis en évidence des différences significatives dans les génotypes pour rs1815739 (*R577X* non-sens), rs1671064 (*Q523R* faux-sens) et rs678397 (variant intronique) entre les bruxeurs et les non-bruxeurs. Les patients présentant un bruxisme auto-déclaré présentaient une surface moyenne des fibres musculaires plus importante pour le type IIA. Les surfaces moyennes des fibres chez les individus avec le génotype CC de type sauvage pour rs1815739 (*R577X*) étaient significativement plus grandes pour les fibres de type IIA [1394,33 m2 (572,77 µm2)] que chez ceux avec les génotypes TC et TT [832,61 µm2 (602,43 µm2) et 526,58 m2 (432,21 µm2). Des résultats similaires ont été retrouvés pour les variants faux-sens et introniques.

**Au total :**

Le polymorphisme nucléotidique rs1815739 (*R577X* non-sens) résulte en une absence de protéine alpha actinine 3, conduisant à une diminution de l'activité contractile rapide, une amélioration des performances d'endurance et une réduction de la masse osseuse et de la densité minérale osseuse. Le génotype muté *ACTN3 577XX* est associé à des surfaces moyennes des fibres de type IIA significativement plus petites. Il protège du bruxisme auto-déclaré. Il semble présenter une tendance protectrice face à la dysfonction temporomandibulaire de type mylagie.

Chez les patients asymétriques, population hautement affectée par la présence d'une dysfonction temporomandibulaire, l'allèle X (muté) du polymorphisme nucléotidique rs1815739 (*R577X* non-sens) est plus élevé lorsque le modelage du condyle est normal, suggérant le rôle adaptatif de ce polymorphisme sur la stabilité condylienne mandibulaire. A*CTN3 577XX* est cependant associé à la présence d'anomalies architecturales sagittales (classe II) et verticales (hypodivergence).

Il semble donc que le génotype A*CTN3 577XX* soit un facteur protecteur du bruxisme auto-déclaré, et à fortiori des dysfonctions temporomandibulaires, et qu'il participe à la stabilité condylienne chez les patients affectés par une dysmorphose dentofaciale. Néanmoins, il est associé à des anomalies architecturales, soulignant le rôle d'adaptations fonctionnelles musculo-squelettiques déséquilibrées sous influences génétiques ou épigénétiques dans l'étiologie des dysmorphoses dentofaciales.

## III. Perspectives

Ces travaux permettent de mettre en évidence *ACTN3 /* l'alpha actinine *3* comme une cible thérapeutique potentielle dans le cadre d'une prise en charge orthodontique et orthognathique personnalisée. Nos résultats histomorphométriques permettent par ailleurs de discuter l'effet thérapeutique de la toxine botulique A et ouvrent le champ à une cible thérapeutique potentielle. *ESR1* et *ENPP1* sont d'autre part de potentiels gènes d'intérêt dans le cadre de la survenue de dysfonctions temporomandibulaires, de résorptions condyliennes postopératoires de dégradations de résultats postopératoires.

### 1. Orthodontie et orthopédie dentofaciale personnalisées

La médecine personnalisée a été définie par le *National Institute of Health* (NIH) et par la *Food Drug Administration* (FDA) comme "une pratique médicale utilisant un profil génétique individuel afin de guider des décisions dans un objectif de prévention, de diagnostic ou de traitement des maladies ». Elle permet d'obtenir « les meilleurs résultats médicaux par le choix de traitements qui fonctionnent bien chez une personne donnée, en fonction de son profil génomique ou avec certaines caractéristiques du sang ou des protéines de surface cellulaire » (Meadows 2005). Dans la pratique orthodontique, l'identification d'un variant clé permettrait notamment, face à un enfant en période de croissance, de prédire s'il a un risque important

d'évoluer vers une dysmorphose dentofaciale spécifique et permettrait d'optimiser sa prise en charge thérapeutique (Carlson 2015; Hartsfield et al. 2017; Vieira 2019). L'identification du génotype *ACTN3 577XX* conduirait alors l'orthodontiste à proposer un suivi plus régulier afin de dépister précocement le développement d'une classe II dento-squelettique, voir à engager plus précocement un traitement à visée orthopédique pour stimuler la croissance sagittale mandibulaire. De la même façon, l'identification du gène *ENPP1* conduirait alors l'orthodontiste à proposer un suivi plus régulier de la morphologie condylienne et du développement d'une asymétrie faciale.

### 2. Chirurgie orthognathique personnalisée : prédiction des complications

Face à une dysmorphose d'indication orthodontico-chirurgicale, l'identification d'un variant spécifique pourrait également permettre d'identifier un sur-risque de développer une dysfonction temporomandibulaire postopératoire, une résorption condylienne ou une dégradation de résultat et d'adapter l'information préopératoire en modulant le risque établi dans la population générale (Annexe 6). Ainsi, d'autres polymorphismes de gènes d'intérêt sont à l'étude dans cette cohorte de patients à l'instar de rs1643821, rs3020318, rs3020377 et rs2077647, 4 polymorphismes nucléotidiques du gène *ESR1* codant pour le récepteur α des œstrogènes et ayant été associé à la survenue de dysfonctions temporomandibulaires. Le récepteur α des œstrogènes est retrouvé chez les sujets masculins comme féminins dans le cartilage intra-articulaire et sur les ostéocytes et joue un rôle de régulateur des médiateurs intracellulaires (Ribeiro-Dasilva et al. 2009). De multiples études d'association ont permis de mettre en évidence une relation significative entre de génotype d'*ESR1* et des symptômes de dysfonction temporomandibulaire (Kim et al. 2010) ou avec une dysfonction temporomandibulaire à type d'ostéoarthrite chez la femme (Kang et al. 2007; Ribeiro-Dasilva et al. 2009; Liu et al. 2014). Dans notre série, nous avons identifié le génotype AA du polymorphisme rs1643821 du gène *ESR1* comme un facteur de risque d'aggravation dysfonctionnelle un an après chirurgie orthognathique (Nicot et al. 2016). De plus, rs1643821 était associé à l'asymétrie de type 4 [différence de hauteur des *ramus* avec déviation du menton du côté ou le ramus est le plus court et inclinaison maxillaire sévère (« asymétrie en forme de C »)] (Chung et al. 2017). Outre la prédictibilité diagnostique, la découverte d'un marqueur génétique de la douleur postopératoire en chirurgie orthognathique nous permet d'entrevoir de nouvelles cibles pharmacologiques thérapeutiques potentielles. En l'occurrence, les voies nociceptives temporomandibulaires médiées par l'estrogène mettent en lumière le rôle potentiel

de la Kétamine, un inhibiteur du glutamate au niveau des récepteurs NMDA, dans la prise en charge de patients sélectionnés. Cette thérapeutique, utilisée sous la forme d'une injection intra-articulaire, semble avoir une efficacité différente selon les formes de dysfonctions articulaires (Ayesh et al. 2008; Alstergren et al. 2010). Une utilisation ciblée en peropératoire d'une chirurgie orthognathique chez les patients avec un génotype muté AA du SNP rs1643821 du gène *ESR1* pourrait être une application thérapeutique à étudier. De la même façon, *ENPP1* pourrait être un gène candidat dans la survenue d'une résorption condylienne postopératoire tandis qu'A*CTN3* pourrait avoir un rôle dans la survenue d'une dégradation de résultat postopératoire.

### 3. Bruxisme et injections de toxine botulique A

Nos résultats histomorphométriques sont particulièrement intéressants afin d'expliquer la physiopathologie des injections de toxine botulique dans les muscles masséters et temporaux pour le traitement du bruxisme. Cette approche thérapeutique produit une réduction de la force musculaire et de la fréquence des événements de bruxisme et est donc largement utilisée dans la gestion du bruxisme pour améliorer la qualité de vie des patients (Almukhtar et Fabi 2019; Fernández-Núñez et al. 2019; Villa et al. 2019; Sendra et al. 2020; Ågren et al. 2020). En effet, les masséters injectés montrent une forte augmentation de la taille des fibres de type IIX, alors que les fibres rapides diminuent d'environ 50% (Korfage et al. 2012). Par conséquent, les injections de toxine botulique entraînent une atrophie d'inactivité associée à une transition de type de fibre, elle-même défavorable au développement du bruxisme. Ces résultats sont similaires à ceux rapportés par Tsai et al. et par Gedrange et al., retrouvant une réduction de la taille des fibres musculaires et une transition des fibres musculaires du type IIA à IIX ou IIB liés à la réduction de la fonction masticatoire chez des rats traités par injections de toxine botulique (Tsai et al. 2012; Gedrange et al. 2013).

Nos résultats mettent en évidence que le génotype A*CTN3 577XX* pourrait être un facteur protecteur du bruxisme auto-déclaré, et à fortiori des dysfonctions temporomandibulaires en participant à la stabilité condylienne, en favorisant un état histomorphométrique défavorable à la survenue du bruxisme autodéclaré. Ces résultats suggèrent qu'*ACTN3*, et par voie de conséquence l'alpha actinine 3, seraient des cibles thérapeutiques potentielles à étudier dans la prise en charge du bruxisme. Toutefois, les différences nycthémérales du bruxisme n'ont pas pu être étudiées ici compte tenu de la temporalité de notre étude par rapport à l'évolution des consensus différentiant le bruxisme du sommeil et le bruxisme de l'éveil.

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# Partie VI : Perspectives de prise en charge mini-invasives des dysfonctions temporomandibulaires intra-articulaires

## I. Généralités sur les dysfonctions temporomandibulaires et leur stratégie générale de prise en charge

Les généralités et la revue de la littérature sur le sujet ne sont pas reprises dans le manuscrit mais ont fait l'objet de plusieurs publications didactiques dans l'Encyclopédie Médico-Chirurgicale de Chirurgie Orale et Maxillo-Faciale :

- Villa S, Nicot R. **Examen clinique et paraclinique de l'appareil manducateur normal et dysfonctionnel.** EMC Chirurgie orale et maxillo-faciale. 2020; Doi : 10.1016/S2352-3999(20)41479-7

- Nicot R, Raoul G, Ferri, J. **Étiopathogénie des dysfonctions temporomandibulaires**. EMC Chirurgie orale et maxillo-faciale. 2020; Doi : 10.1016/S2352-3999(20)41480-3

- Nicot R, Roland-Billecart T, Schlund M. **Pathologies non fonctionnelles de l'articulation temporomandibulaire.** EMC Chirurgie orale et maxillo-faciale. 2021; Doi : 10.1016/S2352-3999(20)41482-7

- R. Nicot, L. Mattei, G. Raoul, V. Tiffreau, J. Ferri, M. Schlund. **Limitation d'ouverture buccale.** EMC Chirurgie orale et maxillo-faciale. 2022; Doi : 10.1016/S2352-3999(21)42356-3
- Nicot R. **Traitements conservateurs, mini-invasifs et chirurgicaux des dysfonctions temporomandibulaires.** EMC Chirurgie orale et maxillo-faciale. 2022; Doi : 10.1016/S2352-3999(21)41481-0

## II. Introduction du projet MaxilloGEL

Les dysfonctions temporomandibulaires sont des myo-arthropathies de l'appareil manducateur, pouvant être responsables de douleur chronique et de retentissement fonctionnel important impactant la qualité de vie des patients. Leur prise en charge est un enjeu de santé publique majeur puisqu'il s'agit de la première cause de douleurs orofaciales dans le monde, représentant 5 à 12% de la population dans les pays industrialisés (Liu et Steinkeler 2013; Schiffman et al. 2014). Parmi les principales dysfonctions temporomandibulaires intra-articulaires, on distingue les déplacements discaux d'une part (dont le caractère peut être réductible ou non), et les arthropathies dégénératives d'autre part (ostéoarthrose ou ostéoarthrite).
La prise en charge de ces DTM est difficile au vu de l'hétérogénéité de la pathologie et des présentations cliniques associées. Une approche multidisciplinaire fondée sur le modèle biopsychosocial de la douleur permet d'appréhender la pathologie dans sa globalité et complexité. Il existe des traitements conservateurs, des traitements mini-invasifs et des traitements chirurgicaux. L'objectif du traitement est la diminution de douleur, l'optimisation des mouvements mandibulaires, dont l'ouverture buccale, permettant d'améliorer la qualité de vie globale des patients (Garrigós-Pedrón et al. 2019; Derwich et al. 2020; Nicot 2022).
Afin de répondre à la nécessité d'un traitement mini-invasif efficace dans la prise en charge des dysfonctions temporomandibulaires intra-articulaires les plus communes (déplacements discaux et arthropathies dégénératives), nous avons proposé le développement d'un hydrogel injectable à base de Chitosan et de Polymères de Cyclodextrine avec système de libération contrôlée d'un principe actif permettant d'incorporer une substance pharmacologique active.

Les hydrogels sont des biomatériaux notamment utilisés pour la délivrance de médicaments ou encore pour la régénération tissulaire sous forme de « Scaffold ». Ils sont constitués à 95% d'eau retenue par des réseaux tridimensionnels de chaines polymères enchevêtrés, qui peuvent être réticulés par des liaisons covalentes (gel chimique irréversible) ou pseudoréticulés par des liaisons faibles (gel physique réversible). L'hydrogel en cours de développement par notre équipe de recherche de l'unité INSERM U1008 est un hydrogel physique composé d'un mélange biocompatible de Chitosan (CHT) et de polymères de Cyclodextrine (PCD).
Le Chitosan est un produit dérivé de la chitine, polymère polysaccharidique naturel retrouvé dans l'exosquelette de certains crustacés et insectes ainsi que dans la paroi cellulaire des

champignons. Il est obtenu après désacétylation partielle de la chitine (degré de désacétylation supérieur à 50%). Le Chitosan présente des propriétés intrinsèques : procicatrisant, hémostatique, antibactérien, et biodégradable (Bhattarai et al. 2010).
Les Cyclodextrines sont des oligosaccharides dérivés de l'amidon. Leur structure cyclique, avec une cavité hydrophobe et une face externe hydrophile, forme des complexes d'inclusion et peut permettre, entre autres application, la libération prolongée de principes actifs. Les Cyclodextrines sont appelés « molécules-cages ». Un PCD, obtenu par réticulation avec l'acide citrique (agent de réticulation) qui confère au polymère un caractère anionique (groupe hydroxyle -COO-), a été développé par l'équipe de l'UMET (Brevet français FR3040883 déposé le 14/09/2015 Numéro de publication internationale : WO2017/046506 A1). Ainsi, deux fractions sont obtenues : soluble (PCDs) et insoluble (PCDi) dans l'eau.
Le CHT est un polymère cationique (groupe amine -NH3+) en milieu acide capable de former un complexe polyélectrolytique par interactions ioniques avec des polymères anioniques tels que les PCD. Une procédure de préparation d'un hydrogel à base de CHT et de polymères anioniques a été breveté par l'équipe de l'INSERM U1008 (Brevet français FR3038318 déposé le 02/07/2015 Numéro de publication internationale : WO2017/001808 A1).

L'utilisation du système CHT-PCD sous forme d'hydrogel (CHT/PCDs/PCDi) permet alors d'associer les caractéristiques de viscoélasticité d'un gel et la libération prolongée locale d'une molécule active (ex. anti-inflammatoire non stéroïdien) et représente donc une option thérapeutique de choix pour le développement d'un hydrogel injectable dans le traitement des principales dysfonctions temporomandibulaires intra-articulaires (déplacements discaux et arthropathies dégénératives). La formulation et l'optimisation de l'hydrogel sont issues des différents travaux réalisés par l'INSERM U1008 en collaboration avec l'UMET. Le développement d'un hydrogel avec l'ajout d'un principe actif au système CHT-PCD fait l'objet d'un dépôt de brevet européen (Dépôt 22306135.9 du 28 juillet 2022 ; N/Référence : B76705EP / D41400 / EL). Les détails de cet hydrogel ne seront donc pas détaillés dans ce manuscrit.

Dans le cadre de l'évaluation de cet hydrogel nu et chargé, un modèle d'ostéoarthrite chez le rat a été développé. Toutes les procédures dans le cadre de ce projet ont été approuvées par le Ministère de l'enseignement supérieur, de la recherche et de l'innovation, référence APAFIS# 25897-2020020715156016 v1 (Annexe 7). Nous présentons ici la stratégie de choix et de mise en place de ce modèle ainsi que les résultats des principaux comparateurs.

## III. Stratégies de prise en charge mini-invasives des dysfonctions temporomandibulaires intra-articulaires par systèmes à libération prolongée

INTRODUCTION : La gestion des ostéoarthrites de l'articulation temporomandibulaire (OATM) est un défi majeur. Des thérapies mini-invasives (basées principalement sur des techniques d'injections intra-articulaires) ont été développées pour permettre une efficacité locale et limiter les effets indésirables systémiques. Cependant, la nécessité de répéter les injections en raison de leur courte durée d'action et les coûts élevés de ces prises en charge ont poussé les chercheurs à développer des systèmes de libération de médicament (SLM). Dans cette revue systématique de la littérature, nous visons à fournir une vue d'ensemble des études qui ont testées les DDS sur un modèle d'OATM.

METHODE : Nous avons recherché sur PubMed les articles publiés de novembre 1965 à mars 2021 sur les SLMs utilisant un modèle d'OATM. Nous avons mis en évidence les différents SLMs et la molécule active employée. La voie d'administration du médicament, le type de modèle, la durée du test et la durée d'efficacité ont été évalués. Pour évaluer la qualité de chaque étude, le biais de protocole a été testé en utilisant QUADAS-2™.

RESULTATS : Sur les 10 études dont le texte intégral a été examiné, quatre ont utilisé un système d'administration à base de poly(acide lactique-co-glycolique). Les autres SLM employaient des hydrogels à base de chitosan, des patchs de micro-aiguilles, des transporteurs lipidiques nanostructurés ou des micelles de poloxamère. L'acide hyaluronique, les anti-inflammatoires non stéroïdiens et les analgésiques ont été utilisés comme molécules actives dans cinq études. Le principal mode d'administration des SLM était l'injection intra-articulaire et le modèle le plus utilisé était le rat.

CONCLUSION : Divers SLM et molécules actives ont été étudiés sur un modèle d'OATM. D'autres travaux utilisant des durées d'essai plus longues sont nécessaires pour valider ces avancées.

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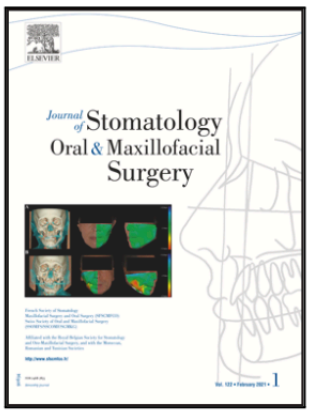


# Systematic review of studies on drug-delivery systems for management of temporomandibular-joint osteoarthritis

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# Systematic review of studies on drug-delivery systems for management of temporomandibular-joint osteoarthritis

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**Systematic review of studies on drug-delivery systems for management of temporomandibular-joint osteoarthritis**


## Abstract

**Introduction**: Temporomandibular-joint osteoarthritis (TMJOA) management is a major challenge. Minimally invasive therapies (based mainly on injections) have been developed to increase local efficacy and limit adverse systemic effects. However, the requirement for repeat injections due to a short duration of action and expensive healthcare costs have pushed researchers to develop, *via* tissue engineering, drug-delivery systems (DDSs). In this literature systematic review, we aim to provide an overview of studies that tested DDSs on a TMJOA model.

**Material and methods**: We searched on PubMed for articles published from November 1965 to March 2021 on DDSs using a TMJOA model. We highlighted the different DDSs and the active molecule employed. Route of drug administration, model type, test duration, and efficacy duration were assessed. To evaluate the quality of each study, a protocol bias was tested using QUADAS-2™.

**Results**: Of the 10 studies that were full text-screened, four used a poly(lactic-*co*-glycolic acid)-based delivery system. The other DDSs employed chitosan-based hydrogels, microneedles patches, nanostructured lipid carriers, or poloxamer micelles. Hyaluronic acid, nonsteroidal anti-inflammatory drugs, and analgesics were used as active molecules in five studies. The main way to administer DDSs was intra-articular injection and the most used model was the rat.

**Discussion**: Various DDSs and active molecules have been studied on a TMJOA model that could aid TMJOA management. Further works using longer test durations are necessary to validate these advances.



**Keywords:** drug-delivery system; tissue engineering; temporomandibular disorders; temporomandibular-joint osteoarthritis; systematic review; minimally invasive therapies

## 1. Introduction

The temporomandibular joint (TMJ) makes the junction between the temporal bone and mandibular condyle. This articulation contains a disk and a surrounding capsule with ligamental and muscular binding that permits jaw mobility. Temporomandibular disorders (TMDs) are myoarthropathies of the manducatory system. TMDs have a considerable impact on the quality of life (QoL) because they induce chronic painful symptoms, muscular spasms, or limit mouth-opening [1]. This complex disease may affect old or young people, disabling daily activities such as talking and chewing, thereby leading to significant repercussions for public health [2].

Among people with TMDs, temporomandibular joint osteoarthritis (TMJOA) is probably one of the main subtypes due to its high clinical prevalence and consequences on the TMJ. TMJOA involves remodeling dysfunction of the joint components, which generates long-term inflammation of the joints, reduction of synovial-fluid volume, and progressive osteocartilaginous degradation [3].

Numerous therapeutic agents have been considered to resolve the problem of the inflammatory process and tissue destruction to limit TMJOA progression [4]. After the failure of non-invasive methods, minimally invasive ones were proposed: intra-articular injection of substances, arthrocentesis, or combination of these two treatments. The most widely used agents are hyaluronic acid (HA) (due to its viscosity-supplementation properties) as well as nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids (for their anti-inflammatory properties). These agents permit a reduction in chronic pain and enhance jaw mobility [5]. However, such therapies have some limitations. The main limitation is the short duration of efficacy, which limits their long-term action against severe disease and necessitates repeat

injections, which are not without consequences: local toxicity due to a high concentration, systemic side effects, QoL reduction, and increases in healthcare costs [6].

To alleviate these problems, prolonged drug-delivery systems (DDSs) have promising potential. Sustained release of a drug into the target site would enhance treatment effects by inhibiting the inflammatory process and increasing TMJ regeneration. The treatment aim is to relieve long-term painful and mechanical symptoms. Usually, DDSs are composed of two distinct types of molecules acting together: a therapeutically active molecule which is released in a prolonged manner by a carrier molecule.

This systematic review of the literature aims to provide an overview of research advances in use of prolonged DDSs for TMJOA management.



## 2. Materials and methods

This systematic review was undertaken following the Declaration of Helsinki 1965 and its later amendments with regard to medical protocol and ethics, as well as Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Owing to the bibliographic nature of our study, the Ethics Review Board of the University of Lille (Lille, France) did not ask for ethical consent. This review has been registered on PROSPERO.

Two authors (F.B. and R.N.) searched independently the PubMed and Web of Science databases to find eligible English written articles for our study. We used two distinct sentences: [((temporomandibular) *or* (TMJ) *or* (TMD) *or* (TMJD)) *and* (drug

delivery system)] and [((temporomandibular) *or* (TMJ) *or* (TMD) *or* (TMJD)) *and* ((prolonged) *or* (sustained)) *and* ((drug) *or* (treatment))]. The literature search was from November 1965 to March 2021. The results were analyzed from the title and abstract of studies dealing with clinical tests (clinical or human) of DDSs for TMJOA management. Tests for measurement of inter-rater agreement in determination of inclusion and exclusion criteria; data extraction and bias assessment resulted in a value of 1.

The exclusion criteria were studies in which: (i) the result was not stated or did not concern a DDS study; (ii) the substance was described without a clinical test; (iii) an articulation other than the TMJ was investigated; (iv) the full text was not available; (v) duplicated data were present. Studies that met the inclusion criteria were evaluated carefully and independently by two authors (F.B. and R.N.) to obtain useful data and bias assessment.

The information extracted from the selected articles was the: (i) the carrier molecule used and its associated active molecule (drug or stem cells); (ii) administration route into the TMJ; (iii) TMJOA model (animals or humans); (iv) duration of the test; (v) duration of efficacious therapy. Each parameter was evaluated further.

We wished to ascertain the quality and scientific impact of each article. Hence, a bias assessment was carried out by F.B. and R.N. using QUADAS-2 tool™. The evaluation was based on several criteria. The most important criterion was the number of participants in each group (which influenced statistical analyses): fewer than five people per group was considered to lead to a “high” risk of a bias, whereas 5–7 participants was as an “intermediate” risk, and ≥8 as “low” risk. The control arms

were also evaluated, with a lack of a control group carrying a high risk of a bias. A "nude" TMJOA model or with a DDS without an active molecule was an intermediate risk, and an active molecule without a carrier molecule (reference treatment) was deemed a low risk. Efficacy criteria were also classified: use of only one criterion (clinical, histology, imaging, or biological) was considered a high risk, an association of two criteria as an intermediate risk, and more than two criteria as a low risk. The test duration was rated. If the study was undertaken during <1 week, it was considered a high risk, between 7 days and 13 days as an intermediate risk, and starting from 14 days as a low risk.

## 3. Results

The search using [((temporomandibular) *or* (TMJ) *or* (TMD) *or* (TMJD)) *and* (drug delivery system)] led to 166 results (115 on PubMed and 51 on Web of Science). The search using [((temporomandibular) *or* (TMJ) *or* (TMD) *or* (TMJD)) *and* ((prolonged) *or* (sustained)) *and* ((drug) *or* (treatment))] elicited 510 results (357 on PubMed and 153 on Web of Science) (Figure 1). Of these 676 articles, 172 duplicates were noted and 494 studies were excluded because of the presence of at least one exclusion criterion: 470 had a result which was not stated or did not concern a DDS study; 16 studies tested the substance without clinical test and 8 studies dealt with an articulation other than the TMJ. Any articles were excluded due to the lack of availability of the full text. Finally, 10 studies were reviewed based on their full text (Table 1).

### 3.1. Bias assessment

Bias assessment was undertaken based on QUADAS-2 tool (Figure 2 and Table 2). Of 10 studies, six showed a high risk of a bias and four an intermediate risk.

### 3.2. Carrier molecule

To counteract the rapid degradation of the active molecule and, thus, reduce the number of intra-articular injections and their undesirable effects, several research teams tested different systems for sustained release. On these 10 articles, four based their DDS on poly(lactic-*co*-glycolic acid) (PLGA) [7–10], two on a microneedle patch [11,12], two on nanostructured lipid carriers [13,14], one on a chitosan-based hydrogel [15] and one on poloxamer micelles [16]. PLGA is a synthetic material which can be used as a carrier in various forms: microparticles [7], nanocapsules [8] or microspheres [9, 10].

### 3.3. Active molecule

Various types of active molecules can be used in DDSs. They can be HA (which is injected) [10,15], anti-inflammatory agents (e.g., naproxen [13], diclofenac [14] and parecoxib [10], or analgesics (e.g., tramadol) [12]. HA and parecoxib were employed in one study [10]. Molecules from natural sources were also employed. For example, 15-deoxy-$^{\Delta 12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$), which slows down the inflammatory process, was used in three studies [8,11,16]. Connective tissue growth factor and transforming growth factor-*β*3 were employed in one study [9] due to their regenerative and tissue-healing abilities. Small interfering ribonucleic acids (siRNAs) silence Fc receptor (Fc*γ*RIII) signaling, and are involved in the secretion of proinflammatory cytokines, and were used in one study [7].

### 3.4. Model creation and drug administration

Nine studies used a small-animal model to evaluate therapeutic efficacy, including rats [7, 8, 10–13, 16] or rabbits [9, 15]. One study used a human model [14].

The product can be delivered to the TMJ area by different means. The most common is intra-articular injection, and six studies used it [7, 8, 10, 13, 15, 16]. The transdermal route by electroporation was employed in one study [14] and diffusion from skin injuries induced by microneedles was used in two studies [11,12]. A surgical procedure to reach the TMJ directly and implant three-dimensional (3D) printed scaffolds was employed in one study [9].

### 3.5. Test duration and efficacy

The test duration ranged from several hours [8, 11], 6–10 days [7, 12–15], to ≥2 weeks [9, 10, 16]. The minimum was 45 min [8] and the maximum was 4 weeks [9].

Interestingly, of 10 treatments, seven demonstrated their efficacy until the end of the test [7–10, 14–16]. For the three other therapies, two were shown to be efficacious for the duration of the test, but were dependent upon the dose used [11] or on the efficacy criteria (behavioural response or inflammatory markers) [12] used, and one was shown to be efficacious for 7 days for a test duration of 10 days [13].

## 4. Discussion

Minimally invasive therapies have two main limitations, they cannot: (i) allow the regeneration of damaged tissues because of their short efficacy; (ii) reduce symptomatology in the long term. These limitations have led to the requirement for repeat injections (which increase the risk of side effects), a reduction in QoL, and a significant cost to public-health systems. However, these limitations have led research teams to develop prolonged DDSs to repair joint injuries and, thus, products which will have an impact on the clinical picture.

Increasingly, tissue engineering of the TMJ is playing an important part in TMD management. In 2008, Mountziaris et al. [6] reviewed intra-articular DDSs. They noted that all systems were studied using animal models but on other joints (e.g., knee), not on the TMJ. In 2018, Dashnyam et al. [17] reviewed emerging systems by focusing on intra-articular injection of biomaterials. They highlighted the important advances in the testing of animal models of TMJOA. However, most of the studies used a naked DDS or only the drug without a carrier. Since 2018, several studies have evaluated the impact of such DDSs with an active molecule in an animal model of TMJOA to validate these promising treatments.



### 4.1. DDSs studied

Various substances are used to create appropriate DDSs. Our systematic review revealed PLGA-based biomaterials to be the most prevalent. This biodegradable synthetic material is used widely in tissue engineering for articular diseases, and its biocompatibility in the TMJ has been demonstrated [18]. PLGA can be used in the form of microspheres or microparticles for extracellular actions [7, 9, 10] or as nanoparticles for intracellular actions [8]. Tarafder et al. [9] incorporated PLGA

microspheres encapsulated with growth factors into 3D-printed scaffolds. Scaffold implantation into the TMJ disks of rabbits improved disk healing thanks to spatially controlled delivery of growth factors. This process is almost identical for poloxamer-based micelles; the morphologic and physicochemical characteristics change but the anti-inflammatory effects are enhanced by this type of DDS [16].

Based on the same mechanism of action, nanostructured lipid carriers are also promising nanocarriers because they allow encapsulation of various substances, such as NSAIDs [13, 14]. Thanks to their biocompatibility and prolonged release of the active molecule, nanostructured lipid carriers can help to reduce local drug toxicity and the volume of drug injected [19].

By disrupting the stratum corneum layer of the skin (the major skin barrier against drug transfer), microneedles allow topical delivery of an active molecule [20]. The main advantages advanced by the two research teams who tested this biomaterial were the non-requirement of intra-articular injections, and creation of a painless system that improved QoL and reduced the stress caused by administration [11, 12].

Chitosan-based hydrogel scaffolds are composed of natural and synthetic biomaterials. Because of their viscoelasticity and biodegradability, hydrogels are compatible with many living tissues, thereby making them a promising system for intra-articular actions [21]. Chitosan is used in a scaffold to release an active molecule (e.g., HA) in a prolonged manner [15].

**4.2. Active molecules released**

Some research teams have tried to develop sustained-release systems by associating active molecules (e.g., HA [10,15], NSAIDs [10,13,14] or analgesics [12]) with the carrier molecule. The main goal is to improve the efficacy of each molecule through administration. We noted the development of new drugs which are not included in minimally invasive therapies. Several pathways have been targeted, such as growth factors [9] (to enhance tissue repair), anti-FcγRIII-siRNA [7] (to avoid the proinflammatory process) or 15d-PGJ2 [8,11,16] (to promote secretion of anti-inflammatory cytokines). A promising option could be an association between several drugs that can act on several features of TMJOA to slow down disease progression and regenerate damaged tissues.

Six studies out of the 10 studied here used intra-articular injection. The transcutaneous route (by microneedles or electroporation) is used rarely in minimally invasive techniques, but has been revived by some research teams [11, 12, 14]. A surgical procedure was rarely used in the studies we reviewed.

Only one study was clinical [14], the others were carried out on rats and rabbits. Because of their anatomy, ease of handling, and low cost, rats and rabbits are often employed to study TMDs. The appropriate model must be chosen so that its use itself does not lead to a bias. The model must take into account parameters such as sex, TMJOA induction, and treatment duration, as described in our previous systematic review [22].

### 4.3. Limitations

One must ensure that a significant difference can be identified during data analyses, so a study must have sufficient statistical power. The latter is characterized by the

size of the groups studied and compared. Therefore, use of too few animals in a study is considered a factor that can lead to a bias. In this systematic review, four studies carried a high risk of a bias or insufficient information [9–11, 13], three had an intermediate risk [8, 12, 16] and three carried a low risk [7,14,15]. The best way to avoid a bias is to anticipate the necessary statistical power before starting analyses and, therefore, to calculate the size of the groups accordingly.

In each of the studies we evaluated, a control group was provided, which eliminated a high risk of a bias. We considered use of a nude TMJOA model or a DDS without an active molecule as an intermediate risk factor because the control group must be the reference treatment or use of the active molecule alone. The main objective of these studies should be to demonstrate the contribution of a sustained-release system. Only three of the studies we evaluated presented a low risk of a bias [12, 14, 15].

Among biology, imaging, clinical data, and histology, two studies [7,16] used more than two criteria for treatment efficacy. The other eight studies were either intermediate [8,9,11–15] or high risk [10]. Indeed, TMJOA affects several parameters including clinical (chronic painful symptoms), histology (cartilage/bone modifications), imaging (inflammation) and biological (inflammatory phenomena) aspects.

Another important feature for DDS evaluation is to increase the efficacy of an active molecule during the test. Three studies revealed a high risk of bias with a test duration <1 week [8, 11, 12] and only three studies had a test duration of ≥2 weeks [9, 10, 16]. The short duration of action of therapies could be circumvented by carrying out preclinical studies.

## 5. Conclusions

Recent advances in tissue engineering have provided promising DDSs and a wide range of active molecules for TMJOA management. The increased number of publications on this subject in recent years testifies to the growing interest of research teams on DDS development. However, some factors (duration of tests) need to be optimized to increase the relevance and evaluation of such products.

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**Figures and legends**

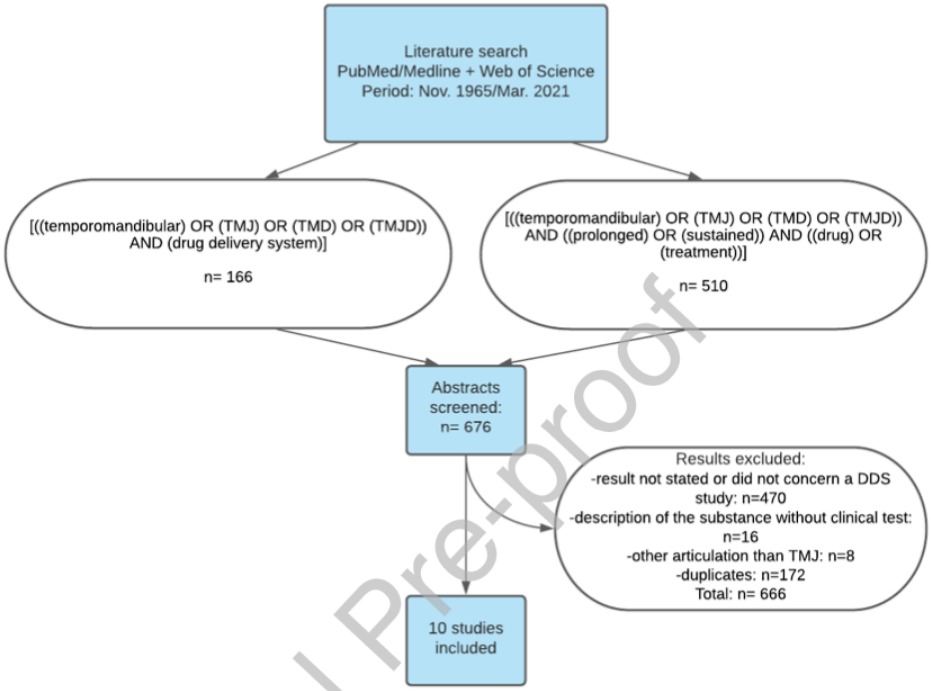
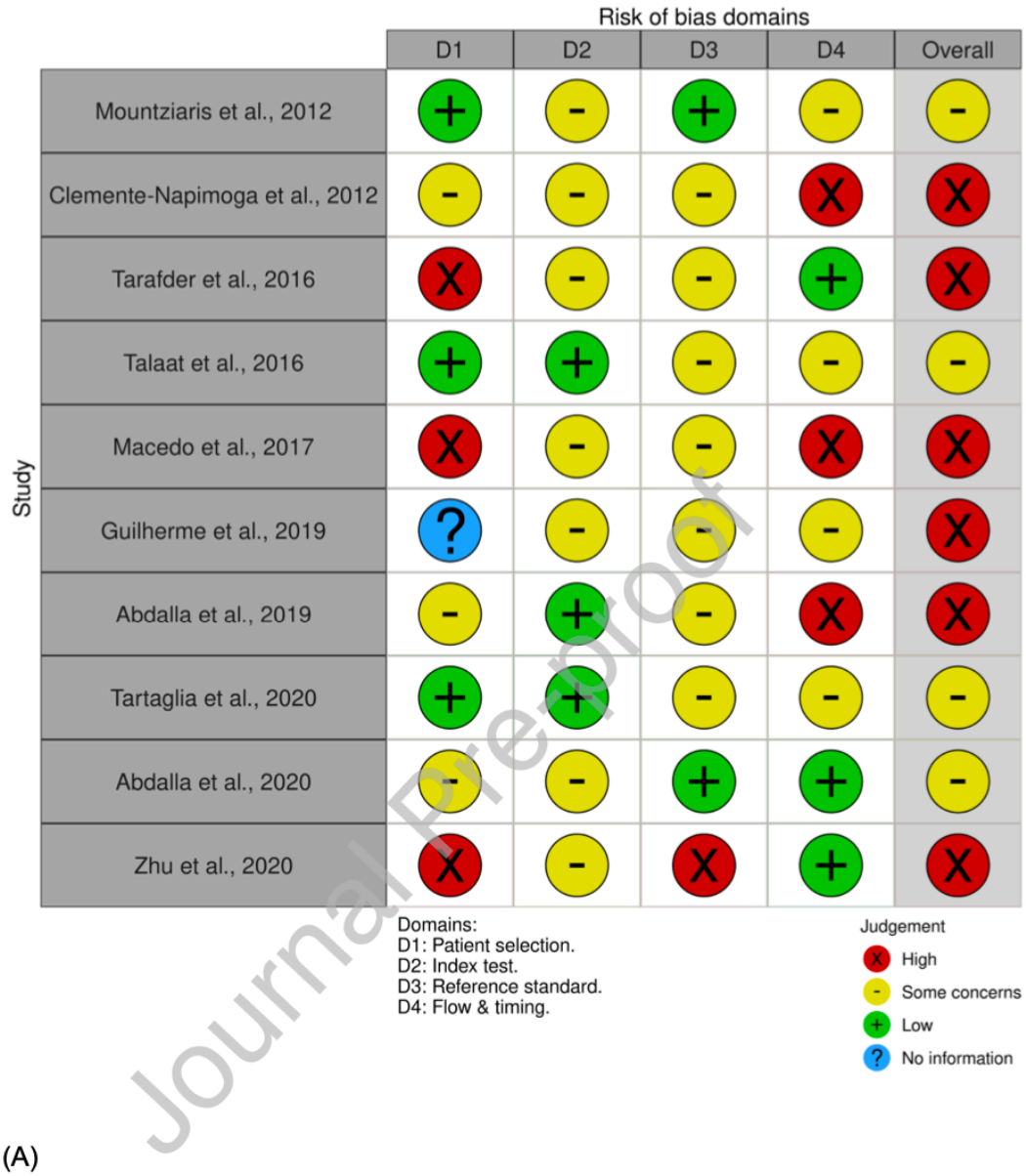
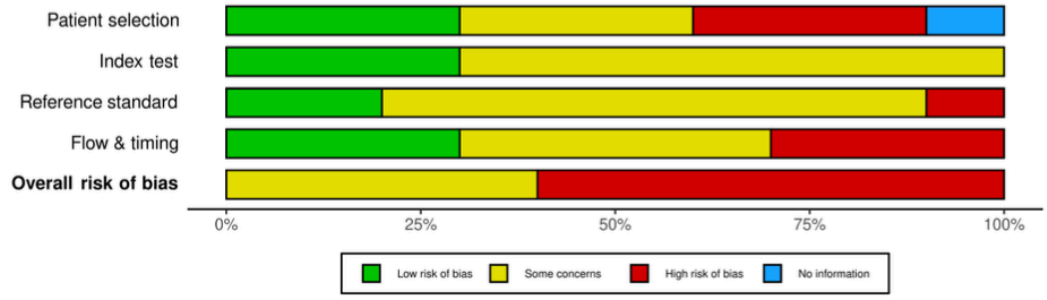



**Figure 1:** Flowchart based on the PRISMA statement.




(A)




(B)

**Figure 2:** Quality assessments using QUADAS-2 tool. A: Table summarizing the different risks of a bias described by the authors in each study. B: Graph representing the proportion of different risk levels found in the studies for each characteristic.

**Table 1:** General characteristics of all studies included in this systematic review.

| References | Carrier molecule | Active molecule | Model | Drug administration | Test duration | Efficacy duration |
|---|---|---|---|---|---|---|
| Mountziaris et al., 2012 | PLGA-based microparticules | Anti-FcγRIII-siRNA-PEI polyplexe | Rat | Intra-articular injection | 9 days | 9 days |
| Clemente-Napimoga et al., 2012 | PLGA-based nanocapsules | 15d-PGJ2 | Rat | Intra-articular injection | 45 minutes | 45 minutes |
| Tarafder et al., 2016 | 3D printed scaffolds with PLGA microspheres | CTGF + TGFβ | Rabbit | Surgery | 4 weeks | 4 weeks |
| Talaat et al., 2016 | Chitosan-based hydrogel | Hyaluronique acid | Rabbit | Intra-articular injection | 7 days | 7 days |
| Macedo et al., 2017 | Microneedles patch | 15d-PGJ2 | Rat | Transdermal | 8 hours | 8 hours depending on dose concentration |
| Guilherme et al., 2019 | Nanostructured lipid carriers | Naproxen | Rat | Intra-articular injection | 10 days | 7 days |
| Abdalla et al., 2019 | Microneedles patch | Tramadol | Rat | Transdermal | 6 days | 2 days : behavioural response<br>6 days: inflammatory markers |
| Tartaglia et al., 2020 | Nanostructured lipid carriers | Diclofenac | Human | Transdermal: electroporation | 7 days | 7 days |
| Abdalla et al., 2020 | Poloxamer micelles | 15d-PGJ2 | Rat | Intra-articular injection | 14 days | 14 days |

| | | | | | | |
|---|---|---|---|---|---|---|
| Zhu et al., 2020 | PLGA-based microspheres | Hyaluronique acid + Parecoxib | Rat | Intra-articular injection | 15 days | 15 days |
| Abreviations: HA: hyaluronic acid; PLGA: poly(lactic-co-glycolic acid); siRNAs: small interfering ribonucleic acids; 15d-PGJ2: 15-deoxy-Δ12,14-prostaglandin J2; CTGF: connective tissue growth factor; TGFβ3: transforming growth factor β3 | | | | | | |

**Table 2:** Table summarizing the different criteria for each study used for realization of the bias assessment.

| References | Number of subject per group | Controle | Effectivness criterias | Test duration |
|---|---|---|---|---|
| Mountziaris et al., 2012 | 9 | CFA injection | Meal pattern<br>Immunohistochemistry<br>Inflammatory cytokines<br>Protein expression | 9 days |
| Clemente-Napimoga et al., 2012 | 6 | 15d-PGJ2 injection | Behavioral nociception response<br>Inflammatory markers | 45 minutes |
| Tarafder et al., 2016 | 4 | Scaffolds with empty microspheres | Histological analysis<br>Morphological analysis | 4 weeks |
| Talaat et al., 2016 | 30 | HA injection | Histological analysis<br>HA concentration | 7 days |
| Macedo et al., 2017 | 4 | 15d-PGJ2 application/injection | Behavioral nociception response<br>Inflammatory markers | 8 hours |
| Guilherme et al., 2019 | Unspecified | Carrageenan injection | Leukocytes migration<br>Inflammatory cytokines | 10 days |
| Abdalla et al., 2019 | 5 | Tramadol injection | Behavioral nociception response<br>Inflammatory markers | 6 days |

| | | | | |
|---|---|---|---|---|
| Tartaglia et al., 2020 | Electroporation 22<br>Control 37 | Corticosteroids injection | Clinical evaluation: MIO, VAS, EMG | 7 days |
| Abdalla et al., 2020 | 6 | Formalin injection | Behavioral nociception response<br>Leukocyte migration<br>Inflammatory markers | 14 days |
| Zhu et al., 2020 | Experimental 21<br>Control 3 | Forced mouth openning | Inflammatory markers | 15 days |
| HA: hyaluronic acid; MIO: maximal incisal mouth opening; VAS: pain visual analog scale; EMG: electromyography;<br>CFA: Complete Freund's Adjuvant | | | | |

## IV. Développement d'un modèle animal pour le développement d'un hydrogel avec système à libération prolongée de substance pharmacologique active

### 1. Revue systématique de la littérature des modèles d'ostéoarthrite temporomandibulaire chez le rat pour l'étude des systèmes à libération prolongée de substance pharmacologique active

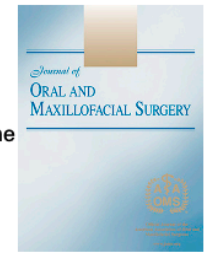
INTRODUCTION : Le développement de thérapies mini-invasives pour la prise en charge de l'Ostéoarthrite de l'articulation temporomandibulaire (OATM) s'est concentré sur l'injection de médicaments anti-inflammatoires non stéroïdiens afin d'éviter les effets indésirables systémiques rencontrés lorsque ces substances sont administrées par voie orale. Par conséquent, nous avons effectué une revue systématique pour répondre à la question suivante : "Quelle méthode d'induction d'un modèle de douleur liée à l'ostéoarthrite temporomandibulaire chez le rat entraîne des symptômes douloureux prolongés, permettant une évaluation optimale d'un système de libération de médicaments (SLM) avec une libération prolongée ?"

METHODE : En suivant les directives PRISMA, nous avons recherché dans MEDLINE les articles publiés de 1994 à juillet 2020 sur un modèle d'OATM chez le rat. Nous avons identifié les moyens d'induction de la douleur et d'évaluation de la nociception. Nous avons évalué les biais du protocole en utilisant une adaptation de l'outil QUADAS-2. La sélection des animaux, la méthode standard de référence d'évaluation de la douleur, l'applicabilité d'une évaluation statistique, ainsi que le déroulement de l'étude ont été évalués.

RESULTATS : Sur les 59 articles complets que nous avons examinés, 41 n'ont effectué aucune évaluation de la douleur après les 7 premiers jours suivant l'induction du modèle de douleur liée à l'OATM. Nous avons finalement identifié 18 modèles de douleur liée à l'OATM à long terme. La douleur était induite par l'injection dans l'articulation temporomandibulaire de substances toxiques, le plus souvent l'adjuvant complet de Freund (50 µg par 50 µl), le formol à diverses concentrations ou l'iodoacétate monosodique (50 mg par 50 µl), ou par des méthodes physiques. Peu d'études ont rapporté des données sur la douleur après 21 jours de suivi. L'hétérogénéité des méthodes d'induction, des méthodes d'évaluation de la douleur et dans le déroulement des études a empêché une méta-analyse.

CONCLUSION : La douleur étant l'un des principaux symptômes de l'OATM, les protocoles des études expérimentales devraient inclure une évaluation de la douleur à long terme.

# Journal Pre-proof




Systematic review of rat models with temporomandibular osteoarthritis suitable for the study of emerging prolonged intra-articular drug delivery systems

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# Systematic review of rat models with temporomandibular osteoarthritis suitable for the study of emerging prolonged intra-articular drug delivery systems


## Abstract:

*Purpose*

Development of minimally invasive therapies for temporomandibular joint osteoarthritis (TMJOA) has focused on drug intra-articular injections to avoid the systemic adverse effects experienced when these substances are administered orally. Therefore, we performed a systematic review to answer the question "Which method of induction of a TMJOA-related-pain model in rats leads to prolonged painful symptoms, allowing the best assessment of a sustained drug delivery system?"

*Materials and Methods*

Following the PRISMA guidelines, we searched MEDLINE for papers published from 1994 to July 2020 on a TMJ arthritis model using rats. We identified the means of pain induction and of nociception assessment. We assessed protocol bias using an adaptation of the QUADAS-2 tool. Animal selection, the reference standard method of pain assessment, applicability of a statistical assessment, and flow and timing were assessed.

*Results*

Of the 59 full papers we reviewed, 41 performed no pain assessment after the first 7 days following induction of the TMJ-related pain model. We eventually identified 18 long-term TMJOA-related pain models. Pain was induced by injection of toxic substances, most commonly Freund's complete adjuvant (50 $\mu$g per 50 $\mu$l), formalin at various concentrations, or monosodium iodoacetate (50 mg per 50 $\mu$l), into the TMJ, or by physical methods. Few

studies reported data on pain after 21 days of follow-up. Heterogeneity of induction methods, pain assessment methods, and flow and timing biases precluded a meta-analysis.

*Conclusion*

Given that pain is 1 of the main symptoms of TMJOA, experimental study protocols should include long-term pain assessment.



**Keywords:** pain; osteoarthritis; temporomandibular joint disorders; models, animal; rats; drug liberation

**Introduction:**

Temporomandibular disorders (TMD) are a significant public health problem affecting approximately 5 to 12% of the general population.[1] This group of heterogeneous musculoskeletal disorders is characterized by either regional pain in the preauricular or facial area or by jaw movement limitation. Subtypes of TMD include pain-related disorders, such as myalgia, myofascial pain with or without pain referral, and arthralgia; and disorders associated with the temporomandibular joints (TMJ), such as internal derangements and degenerative joint disease. Either type results in pain and disability, impacting daily activities, psychosocial functioning, and altering the quality of life. DJD, also known as osteoarthrosis or osteoarthritis (TMJOA), is 1 of the most common taxonomic subtypes of TMD.[2] The prevalence of TMJOA varies greatly, clinical evidence of the disease being observed in 2 to 16% of the population, and structural involvement of the TMJ can be found in 35 to 94% of the patients with at least 1 symptom.[3] This entity is clinically associated with pain in the pre-auricular area with or without associated earache, pain during palpation, coarse crepitus with or without clicking, and limited mobility of the jaw.[2] Its diagnosis is mainly based on radiographic features, including pinching of the joint space, cortical bone resorption, subchondral cysts and geodes, subchondral bone sclerosis, and osteophyte formation.

TMJOA is characterized by progressive cartilage and bone destruction leading to joint inflammation. Therefore, pharmacologic approaches having paralleled those for symptomatic treatment of osteoarthritis have been developed, including NSAIDs[4] and intra-articular injections into the superior joint space (corticosteroids, hyaluronic acid or platelet-rich plasma from blood) [4–6]. However, use of these agents remains controversial in light of decades of mixed reports of intra-articular injections either accelerating TMJ destruction or triggering regeneration [4,6]. To date, no agents have allowed to reverse the underlying TMJ disease.

Consequently, current pain reduction techniques are effective in the early stages of the disease, but fail to alleviate chronic pain caused by severe degenerative joint disease.

There is a high need for sustained release agents, enabling to reduce pain for a long time without systemic adverse effects, which can be seen with current treatments such as NSAIDs [6]. In this light, the methods of intraarticular drug delivery to the TMJ (nano or microparticles), as well as emerging injectable controlled release systems with potential to improve TMJ drug delivery, were under development by numerous researchers to encourage further research in the development of sustained release systems for both long-term pain management and to enhance tissue engineering strategies for TMJ regeneration [7].

Animal models are a useful tool for understanding the pathophysiological mechanisms underlying TMJ disorders, and for evaluating the efficacy of intra-articular injections. A variety

of animal models have been used to evaluate various aspects of drug delivery to the TMJ, including adverse effects of existing intra-articular formulations and the efficacy of emerging treatments. Rodent models are commonly used in studies focusing on temporomandibular degenerative joint disease and TMJ pain, and at the first step in preclinical studies of TMJ drug delivery systems. Rat models of TMJ inflammation have been developed using a variety of methods ranging from repeated, manual, forced mouth opening (mechanical method), surgical procedures, to intra-articular injection of chemical agents. Various analytical methods, such as non-invasive meal pattern analysis, behavior monitoring, etc., have been published to assess as the results for painful symptoms.[8] However, 1 of the main difficulties consists in obtaining a model facilitating the induction of pain in a sufficiently prolonged manner to evaluate the analgesic effect of a long-term drug delivery system.

This review systematically discusses the rat models of TMJOA-related pain in order to identify the best option for assessing long-term controlled drug delivery systems.

**Materials & Method:**

This systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[9] This study followed the Declaration of Helsinki on medical protocol and ethics. Due to the bibliographic nature of this study, it was granted an exemption in writing by the University of Lille IRB.

*Focused Question*

The research question of this study was "Which method of induction of a TMJOA-related-pain model in rat leads to prolonged painful symptoms, particularly suitable for the assessment of a controlled long-term drug delivery system?"

*Search Strategy*

The search was performed in MEDLINE/PubMed databases, from 1994 to July 2020, using the following terms: "TMJ OR temporomandibular OR TMD", AND "nociception OR pain", AND "model", AND "rat".

Studies were eligible if a TMJ arthritis model using rats was described. Exclusion criteria were as follows: literature reviews or studies only describing the method of induction of the TMJ arthritis model, absence of pain assessment in the TMJ arthritis model, absence of control arm for pain assessment, language other than English, or unavailability of the full paper.

The process of searching and selecting the studies was conducted in duplicate by 2 authors (F.B. and R.N.) working independently. Studies were first screened based on an evaluation of the title and abstract, the potential articles were then carefully assessed according to the eligibility criteria of this review.

*Data Extraction*

First, listed the characteristics of the eligible experimental models were listed out. Sex, breed, and weight (in grams) of rats used in the selected studies were extracted. The method of induction of the arthritis model was described. For each of the chemical induction methods, type, volume, and concentration of the chemical agent for TMJ intra-articular injection were detailed. Similarly, for each of the mechanical induction methods, load, frequency and duration of application were extracted. The main methods of nociception assessment and the main categories of variables evaluated (clinical, biological, histological, radiological, and electrophysiological) were detailed. When a study used multiple assessment methods, we selected only the most reliable if one could be identified. Finally, the aim of each study was listed.

Further, to sort out the suitable methods for assessing controlled long-term drug delivery systems to treat TMD, the focus was set on all the studies presenting symptoms of TMD related pain that was lasting statistically more than 7 days longer than the control arm. The control group could be a group of rats injected with 0.9% NaCl (saline) or a therapeutic group of rats, in which an efficient therapeutic injection relieved the chemical-induced TMJOA-related pain. In addition to the data previously collected, the number of rats of the induction group and the control was extracted, and the longest duration (in days) of detectable TMJ pain caused by the arthritis induction method was compared to the control.

*Assessment of Protocol Bias*

Two authors (R.N. and F.B.) independently evaluated the methodological quality rating to verify the strength of scientific evidence on the selected methods of inducing TMJOA-related pain. Protocol bias assessment was performed using an adaptation of the QUADAS-2 tool. Animal selection, the reference standard method of pain assessment, applicability of a statistical assessment, and flow and timing were assessed.

Animal selection: given the role of sex in pain, we considered studies at high risk of bias when both male and female rats were included, at intermediate risk when only females were included, and at low risk when only males were included.

Reference standard method of pain assessment: we considered studies at high risk of bias when they measured the pain using the animals' meal pattern, at intermediate risk when standardized behavioral assessment such as head flinching or orofacial rubbing was performed, and at low risk when a measure of the threshold value using the head withdrawal test was included.

Applicability of a statistical assessment: we considered the studies at high risk of bias when the statistical analysis was performed on fewer than 5 rats in each group, at intermediate risk when between 5 and 7 rats were used, and at low risk when at least 8 rats were used.

Flow and timing: we evaluated if an appropriate time interval was considered between the induction of the model and the last checkpoint using the reference standard method of pain assessment for evaluating long-term TMJ-related pain. We considered studies at intermediate risk of bias when pain assessment was conducted for less than 21 days, and at low risk of bias when pain assessment was conducted for at least 21 days.



**Results:**

The initial search yielded a total of 174 results (Figure 1). Among them, 115 studies were nonrelevant or were not eligible, since they did not assess nociception, focused on wrong joint, concerned an injured joint, or used an animal other than the rat. Therefore, 59 full papers were reviewed. In order to sort out the suitable methods for assessing controlled long-term drug delivery systems, 41 studies were then excluded because there were no symptoms of TMD

related pain that was lasting statistically more than 7 days longer than the control arm. In the end, 18 papers were analyzed.

*Studies Using a TMJ-related Pain Model*

The results are shown in Table 1. Seven studies included only female rats,[10–16] and 4 included both female and male rats.[17–20] In all other studies, only male rats were included. Wistar rats or Sprague-Dawley rats were used in all but 3 studies, in which females Holtzman rats were used.[12–14]

The mechanical induction method was applied in 5 studies[12–14,17,21] and chemical 1 in the remaining 54. No study using surgical technique for inducing an osteoarthritis model was found.

All cases of the mechanically induced model consisted of applying a repeated daily mouth opening using a force of 2 N or 3.5 N. In all cases, the load was daily applied under general anesthesia for 60 minutes for 7 consecutive days. Interestingly, in 3 of the 4 studies using a Holtzman rat, the model induction method was mechanical.[12–14]

Several chemical agents have been used to induce TMJOA. Freund's adjuvant is an emulsified mineral oil solution of antigen with immunopotentiator characteristics. Its complete form, Freund's Complete Adjuvant (CFA), is composed of inactivated and dried mycobacteria (mainly *Mycobacterium tuberculosis*). Injection of CFA was used in 33 of 54 studies.[11,15,16,18,20,22–49] Among them, the standard dose used to induce the model was in most cases 50 $\mu$g of CFA in a 50 $\mu$l volume.[15,24,25,28–30,34–37,41,42,46,47] The volume and concentration of injected CFA is, however, quite variable in other studies.[16,18,20,22,23,26,31–33,38–40,43–45,48–50] Except the study of Ivanusic et al. [39], in which TMJ injection was made using 1 $\mu$g of CFA in a 2 $\mu$L volume, the CFA dose ranged from 15 $\mu$g to 250 $\mu$g with a volume varying from 15 to 100 $\mu$L. In 9 studies, osteoarthritis was induced by injecting formalin into the TMJ at a

concentration varying from 0.5 to 5%.[51–59] Monosodium iodoacetate (MIA) is an inhibitor of glycolysis, which disrupts chondrocyte metabolism and produces cartilage degradation.[10,11,19] Other chemical agents used were carrageenan,[60–63] mustard oil,[64,65] zymosan,[66,67] and methylated bovine serum albumin.[27,68]

Regarding the main method of nociception assessment, a threshold value was used in 34 of 59 studies.[10–15,17,19,21,23–26,28–37,39,41,42,44,46,50,60,63,66–68] With the exception of 1 study using the paw withdrawal test,[44] the head withdrawal test was used in all cases. The threshold value was obtained with a Von Frey[10,12–15,17,19,21,23–26,28–36,41,44,46,66–68] or Semmes-Weinstein[37,39] or unspecified digital aesthesiometer.[11,42,50,60,63] Behavioral evaluation was the main assessment method in 18 of 59 studies.[13,20,22,27,45,51–59,61,62,64,65] Behavioral assessment included head flinching,[27,51,53,54,57,59,61,62,64,65] orofacial rubbing,[20,22,27,45,51–59,61,62,64,65] or chewing.[51,64] In 1 case, a rat grimace scale was performed by scoring facial expression.[13] Meal pattern was used as the pain assessment method in some studies.[16,18,20,38,40,47,48,53] Sleep disturbance has also been used as an assessment method in 3 studies, all of which were from the same research team.[20,43,49]

*Studies Evaluating Long-term TMJ-related Pain*

The results concerning the long-term TMJOA-related pain model are shown in Table 2. Among the 18 studies analyzed, 3 used only female rats,[10–12] and 1 used both female and male rats.[19] In all other studies, only male rats have been used.[21,23,24,26,29–31,33,34,36,40,44,57,68] In all studies, they were Wistar rats[21,24,29,57,68] or Sprague-Dawley rats,[10,11,19,23,26,30,31,33,34,36,40,44] with the exception of 1 study using Holtzman rats.[12]

The TMJOA induction method was mechanical in 2 studies[12,21] and chemical in the remaining 16.[10,11,19,23,24,26,29–31,33,34,36,40,44,57,68]

Both cases of the mechanical model consisted of applying a repeated daily mouth opening

using a 2-N[21] or 3.5-N.[12] In all cases, the load was applied for 1 h daily for 7 consecutive days.

As mentioned before, 2 chemical agents (CFA and MIA) have been mainly used to induce the pain model. TMJ injection of CFA was used in 11 of 18 studies.[23,24,26,29–31,33,34,36,40,44] In most of the cases, animals were injected with a volume not exceeding 50 $\mu$L into the TMJ. Only 2 studies used a volume of 60 $\mu$L[33] or 100 $\mu$L of CFA solution[23]. The injected CFA concentration varied from 0.5 $\mu$g/$\mu$L[26,31,44] to 1 $\mu$g/$\mu$L.[23,24,29,30,33,34,36] Only 1 study used a concentration of 5 $\mu$g/$\mu$L.[40] TMJ injection of MIA was used in 3 studies.[10,11,19] In 2 of them, a solution containing 50 mg of MIA in a 50 $\mu$L volume was injected into the TMJ.[10,11] The third study compared 2 other doses (80 mg/mL, 16.6 mg/mL) of MIA.[19] Other chemical agents used were methylated bovine serum albumin (10 $\mu$g in a 10 $\mu$L volume)[68] and formalin (45 $\mu$L volume of formalin 1.5%).[57]

Regarding the method of nociception assessment, a threshold value was used in all the studies except 2, which used behavioral assessment[57] or meal pattern.[40] In most of the cases, the threshold value was obtained by using a head withdrawal test obtained with a von Frey aesthesiometer.

The number of rats in each group (model induction or control group) was less than 5 in 3 studies[11,33,34] and between 4 and 8 in 9 studies.[21,23,24,26,30,36,57,68] Only 6 studies compared groups each containing at least 8 rats.[10,12,19,29,40,44]

Finally, 6 studies described a TMJOA-related pain model with long-term pain lasting at least 3 weeks.[10,11,23,30,40,68] Three of them used CFA[23,30,40] for TMJ injection, 2 MIA,[10,11] and 1 methylated bovine serum albumin.[68]

*Bias Assessment of the Selected Studies*

The results are listed in Figure 2. Based on the QUADAS-2 tool, 2 studies had a low risk of

bias,[29,44] 11 studies had an unclear risk of bias,[10,12,21,23,24,26,30,31,36,57,68] and 5 showed a high risk of bias.[11,19,33,34,40]

Given the multiplicity of induction methods, the pain assessment method and the flow and timing biases, it was not possible to perform a meta-analysis.

## Discussion:

*Summary of Evidence*

The experimental models of TMJ pain simulate either the symptoms or signs of TMJ pain mainly through the development of arthritis or osteoarthrosis, by using chemical and inflammatory agents, mechanical TMJ loading, or surgical procedures.[8] Although TMJ disorders have a complex taxonomy, all share common traits such as inflammation and pain.[1] Therefore, current therapeutic research axes focus on the development of pharmacological substances contributing to locally reducing inflammation and pain.[4] The specifications of good medication candidates must include the control of pain-related symptoms with low systemic adverse effects and long-term local efficacy. Considering these reasons, research has been directed toward the development of drug candidates combining with a sustained release system. Validation of such treatments requires the use of a specific experimental model that manifests pain-related symptoms long enough to allow an assessment of these long-term drug delivery systems. This systematic review thus focused on experimental models of TMJOA-related pain. Among the 18 studies selected, we identified some mechanical and chemical methods inducing a TMJOA-related pain model. Similarity in the profiles between these mechanical and chemical models suggests that they may induce similar molecular mediators and/or structural changes leading to painful symptoms.[4,8] We finally highlighted 6 studies that achieved an induction of joint pain for at least 3 weeks, but 4 of them had unclear risk of bias

and 2 had a high risk of bias. In all cases,[10,11,23,30,40,68] the induction method was chemical, mainly by the injection of CFA[23,30,40] or MIA.[10,11] The injection of these toxic substances into the TMJ has the advantage of being simple and reproducible. The recognition of specific landmarks previously described easily allows the operator to establish the location of the TMJ.[11,69] Moreover, unlike the surgical induction method, these chemical induction methods generally do not alter the joint anatomy.[70] On the other hand, it has been reported that injection of a toxin such as CFA into the TMJ causes morphological and molecular changes in the contralateral joint, suggesting that the unilateral injection of a toxic chemical agent is sufficient to induce a TMJOA-related pain model,[71] although the uni- or bilateral status of the TMJ injection is poorly documented in these studies. Thus, the injection of toxic substances into the TMJ seems to be able to establish a valuable model for subsequently performing the joint injections of medication candidates carried by a sustained drug delivery system. The main disadvantage of the chemical method could be that, a chemical substance is introduced into the TMJ, which could further interact with the therapeutic substances to be tested or local environment, either by the agent itself directly modifying these therapeutic substances due to its intrinsic properties, or by the solvent in the injection mixture interacting with the intra-articular environment.

*Limitations*

This systematic review assessed protocol bias using an adaptation of the QUADAS-2 tool. Evaluation of animal selection has shown that 4 of 18 long-term pain assessment studies included at least some female rats.[10–12,19] The role of gender in the occurrence of human TMD has been investigated for many years.[72] The intensity of painful symptoms appears to be greater in women for many anatomical locations, including the TMJ. In addition, sex differences in osteoarthritis prevalence and incidence have been shown, with females

generally at a higher risk for developing knee or hand osteoarthritis, particularly after menopause.[73] In a TMJ-osteoarthritis-related pain model, female rats demonstrated a similar spread of tactile hypersensitivity at the lower dose of MIA, whereas male rats did not develop ongoing pain or spread of tactile hypersensitivity outside the area of the ipsilateral TMJ.[19] It suggests that females have a higher susceptibility to develop ongoing pain and central sensitization compared with male rats, a susceptibility that is not due to differences in MIA-induced joint pathology. Therefore, studies evaluating TMJOA-related pain should include only rats of the same gender in order to avoid this selection bias. Given the likely hormonal character and the intra- and interindividual variability of this parameter, the choice should focus on male rats.

Several methods have been proposed in the literature to assess orofacial pain. We identified 4 types of assessment methods: meal patterns, behavioral assessment, sleep patterns, and the threshold value measurement by using the head withdrawal test. The 6 selected long-term TMJOA-related pain models used the paw or head withdrawal test, behavioral assessment, or meal patterns to objectify the pain. These 3 methods can be used with a quantitative dimension. Meal patterns differentiate the ingested meal quantity, frequency, and duration, and the animal stool.[40] Behavioral assessment can be measured by adding the sum of head flinching or orofacial rubbing during a defined lapse of time or use a standardized approach with a quantitative score such as the rat grimace scale.[13] The head withdrawal test classically uses an aesthesiometer, which is applied on the TMJ and leads to rat head withdrawal at a threshold value when the device pressure induces pain. However, the specificity of these 3 different methods seems different. While the withdrawal test appears to be quite specific for pain assessment, the behavioral assessment and the meal patterns could both be influenced by external factors such as stress or illness. Moreover, behavioral assessment is expected to lead to observer bias even if video-recording of rats is used to reduce it.[74] In addition, the

possibility of using an electronic aesthesiometer to perform the head withdrawal test considerably improves the reliability of the threshold value obtained. These are probably the reasons that most of the studies used the head withdrawal test as the main assessment method. Nevertheless, all studies fail to describe the daily rhythm of the measures performed, even though nociception exhibits a robust daily rhythmicity in rats: sensitivity to pain is highest late in the dark phase of the light-dark cycle and lowest at the light-dark transition.[75] It is likely that taking into account the circadian rhythm of pain would change the response to most of the pain assessment methods.

One of the major concerns in the evaluation of these models was the quality of the statistical method. A study design sequentially requires to decide the experimental setting, identify the most appropriate statistical tests, and calculate the sample size that guarantees identifying an expected outcome as statistically significant with appropriate power level. Power analysis must therefore be calculated to ascertain the number of animals per group. Usually, to calculate the number of animals required, one must know the effect size (the estimated difference between the 2 groups), the estimated standard deviation (for continuous variables), the desired power (usually 80%), and the significance level (usually 5%; $p < 0.05$). Neglecting to identify the appropriate sample size in the planning stage or having misestimated the variables necessary to calculate it may potentially compromise the results, since the sample size could turn out to be too small when testing the outcomes in the final statistical analysis.[76] It was quite surprising to see through this systematic review some studies carrying out statistical comparisons on 2 arms each containing 3 to 4 rats. Thereby, we selected a cut-off according to the minimum value necessary for the applicability of statistical tests to evaluate the statistical bias. In total, 12 of the 18 studies did not meet the sufficient conditions to perform relevant statistical tests.[11,21,23,24,26,30,31,33,34,36,57,68] In addition, no study has detailed the calculation of the statistical power required. Therefore, it seems that this point

remains 1 of the essential criticisms because even if the most relevant evaluation method was chosen in most studies, few have carried out statistical tests with numbers allowing relevant comparisons. On the other hand, we considered the flow and timing bias, pointing out studies with an inappropriate time interval between the induction of the model and the last checkpoint using the reference standard method of pain assessment to evaluate long-term TMJ-related pain. Consequently, the studies concerned might not have demonstrated pain for at least 3 weeks given their lack of long-term evaluation of this parameter.

In conclusion, the results of this systematic review showed that the chemical method is currently a valuable option to obtain a long-term TMJOA-related pain model. CFA (50 $\mu$g of CFA in 50 $\mu$L) and MIA (50 mg of MIA in 50 $\mu$L) are the 2 main chemical agents injected into the TMJ to induce this specific condition. The practical implication of this finding is that these methods seem both to be the best options for evaluating sustained drug delivery systems. Nevertheless, it appears that induction protocols for TMJOA focused mainly on long-term histopathologic assessment, and few clinical data are available after 21 days of follow-up. Given that pain is 1 of the main symptoms of TMJOA and the future direction of mini-invasive treatments, experimental protocols should include long-term pain assessment in order to allow the evaluation of sustained drug delivery systems.

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| Study | Rat Sex | Rat Breed | Weight | Method of Model Induction | Main Method of Nociception Assessment | Parameters Evaluated | Aim of the study |
|---|---|---|---|---|---|---|---|
| Abdalla et al., 2020 | Males | Wistar | 200-250g | 45µl Fomalin 1.5% | Behaviour: head flinching and orofacial rubbing | Clinical + Biological + Histological | To assess the viability, effectiveness and longevity of a PL-based micellar system containing 15d-PGJ2 (PL-15d-PGJ2) in a formalin-induced inflammatory pain model |
| Caminski et al., 2020 | Males | Wistar | 250-350g | 50 µl CFA | Behaviour: orofacial rubbing | Clinical + Histological | To evaluate the antinociceptive effects of the CTK 01512-2 toxin with acute, inflammatory, chronic, and neuropathic orofacial pain models, as well as measuring the in vitro and in vivo glutamate levels |



*Table 1*





| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Cornelison et al., 2020 | Males + Females | Sprague-Dawley | 350-500g (Males) 250-300g (Females) | Mouth openning: 2N or 3,5N, 1h every day, 7 days | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological | To test the hypothesis that exposure of animals to the identified TMD risk factors of neck muscle tension, prolonged jaw opening, and female gender would promote persistent sensitization of trigeminal neurons and enhanced nociception indicative of chronic TMD |
| Ribeiro et al., 2020 | Males | Wistar | 180-250g | 50µl of Formalin 1.5% | Behaviour: head flinching and orofacial rubbing | Clinical + Biological | To evaluate the antinociceptive and antiinflammatory effects of Caulerpa racemosa |
| Sperry et al., 2020 | Females | Holtzman | 243-285g | Mouth openning: 2N or 3,5N, 1h every day, 7 days | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To investigate hypoxia-inducible factors and hypoxia after TMJ loading inducing sustained (3.5 N loading) or resolving (2 N loading) pain |
| de Sousa et al., 2019 | Males | Wistar | 200-250g | 10 µg mBSA in 10 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological | To investigate the morphological changes of the synovial membrane during the development of TMJ arthritis, as well as the participation of canonical Wnt and NF-κB pathways in the progression of this chronic disease. |
| Ferrara-Jr et al., 2019 | Males | Sprague-Dawley | 250-370g | 100 µg CFA in 100 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological | To evaluate the effects of Photobiomodulation, as well as the mechanisms involved |
| Garattini et al., 2019 | Males | Wistar | 160-220g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological | To investigate whether the endogenous Hydrogen sulfide production pathway contributes to arousal and maintenance of orofacial inflammatory pain |
| Jin et al., 2019 | Males | Sprague-Dawley | 220-280g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Histological | To examinate morphologic alterations of satellite glial cells in trigeminal ganglion following TMJ inflammation and changes in Connexin 43, glial fibrillary acidic protein and sodium channel 1.7 expression |
| Sannajust et al., 2019 | Males + Females | Sprague-Dawley | 225-275g Males / 175-200g Females | MIA 16.6mg/ml or 80mg/ml | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To characterize sex differences in development of ongoing pain and central sensitization |
| Scarabelot et al., 2019 | Males | Sprague-Dawley | 250-300g | 25 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To investigate the effect of Transcranial Direct Current Stimulation, a non-pharmacological therapy, on local mechanical hyperalgesia, and remote thermal hyperalgesia |
| Zhang et al., 2019 | Females | Sprague-Dawley | 198-271g | 0.5mg MIA in 50µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Histological | To evaluate the effects of weekly intra-articular injections of mesenchymal stem cells exosomes in model of TMJOA, and to investigate the molecular mechanism of exosome-mediated cellular processes and restoration of matrix homeostasis in TMJ repair and regeneration |
| Alves et al., 2018 | Males | Wistar | 180-240g | 50µl Fomalin 1.5% | Behaviour: orofacial rubbing | Clinical + Biological + Histological | To investigate the unexplored anti-nociceptive and anti-inflammatory efficacy of Abelmoschus esculentus lectin in model of formalin-induced temporomandibular joint inflammatory hypernociception |
| Bonfante et al., 2018 | Males | Wistar | 150-250g | mBSA + CFA | Behaviour: head flinching and orofacial rubbing | Clinical + Biological | To investigate if a persistent model of albumin-induced arthritis hypernociception in the TMJ results in the release of pronociceptive factors by microglial cells located in the trigeminal subnucleus caudalis associated with sensitization of central nervous system |
| Ito et al., 2018 | Males | Sprague-Dawley | 200-300g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To determine the involvement of TNF-α signaling in the trigeminal ganglion in the mechanical hypersensitivity of the masseter muscle during TMJ inflammation |
| Santos et al., 2018 | Males | Wistar | 250g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological | To evaluate the hypothesis that TMJ inflammation-induced hyperalgesia and allodynia responses are mediated by endogenous hydrogen sulfide |
| Sperry et al., 2018 | Females | Holtzman | 268 +/- 21g | Mouth openning: 0N, 2N or 3,5N, 1h | Head withdrawal (Von Frey aesthesiometer) + Rat | Clinical + Biological + | To assess Rat Grimace Scale ability to detect TMJ pain induced using repeated TMJ loading that produces moderate osteoarthritic pathology in the joint |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | every day, 7 days | Grimace Scale | Histological | |
| Alves et al., 2017 | Males | Wistar | 160-220g | Zymosan (2mg in 40µl) | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological | To investigate the unexplored anti-nociceptive and antiinflammatory effects of strontium ranelate on the zymosan-induced inflammatory hypernociception in the TMJ by evaluating the IL-1-β and TNF-α levels after strontium ranelate treatment |
| Koop et al., 2017 | Males | Sprague-Dawley | 350-500g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological | To investigate the role of neuropeptide calcitonin gene-related peptide and protein kinase A in promoting cellular changes in the spinal trigeminal nucleus and trigeminal ganglion, and nociceptive response to mechanical stimulation |
| Kartha et al., 2016 | Females | Holtzman | 245 +/- 16.2g | Mouth openning: 2N or 3,5N, 1h every day, 7 days | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To adapt an existing model of mechanically induced TMJOA, to induce persistent orofacial pain by altering only the jaw-opening force, and to measure the expression of common proxies of TMJOA, including degradation and inflammatory proteins, in the joint |
| Lacković et al., 2016 | Males | Wistar | 300-330g | 50 µl CFA | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To investigate the reactivity of cranial dura to trigeminal pain and the mechanism of botulinum toxin type A action on dural neurogenic inflammation |
| Scarabelot et al., 2016 | Males | Sprague-Dawley | 250-300g | 25 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical | To evaluate the effect of acute melatonin administration in the nociceptive response and central biomarkers levels in a chronic inflammatory orofacial pain model |
| Magni et al., 2015 | Males | Sprague-Dawley | 200-250g | 60 µl CFA in 60µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To understand the role of specific P2Y receptors in trigeminal ganglion-related pain |
| Cady et al., 2014 | Males | Sprague-Dawley | 300-400g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To investigate the role of orexins in modulation of trigeminal nerve activation in response to acute and prolonged inflammation of the TMJ, which occurs in TMJ disorders |
| do Val et al., 2014 | Males | Wistar | 160-220g | Zymosan (2mg in 40µl) | Head withdrawal (Von Frey aesthesiometer) | Clinical + Histological | To investigate the unexplored antinociceptive and anti-inflammatory efficacy of T. toxicaria in the model of zymosan-induced TMJ inflammatory hypernociception |
| Cavalcante et al., 2013 | Males | Wistar | 180-200g | 10 µl of Carrageenan 5% | Head withdrawal | Clinical + Biological + Histological | To highlight the role of NMDA receptors in the hypernociceptive process in the TMJ region |
| Hatch et al., 2013 | Males | Sprague-Dawley | 200-250g | 50 µg CFA in 50 µl | Head withdrawal (Semmes-Weinstein aesthesiometer) | Clinical + Biological + Radiological | To investigate whether there was a change in the proportion or intensity of hyperpolarization-activated cyclic nucleotide-gated channel immunoreactivity in TMJ primary afferent neurons following inflammation |
| Li et al., 2013 | Males | Sprague-Dawley | 180-225g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological | To further explore the mGluR5 involvement in inflammatory pain of the trigeminal system, particularly in the TMJ, to determine the expression of mGluR5 protein, and to investigate whether CFA-induced TMJ inflammation causes changes in the levels of mGluR5 protein expression in the trigeminal ganglion |
| Bi et al., 2012 | Females | Sprague-Dawley | 200-220g | 50 µl CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To examine whether TMJ inflammation could influence the expression of Nav1.7 in trigeminal ganglion and whether blocking Nav1.7 function in trigeminal ganglion could attenuate the hyperalgesia of TMJ |
| Garrett et al., 2012 | Males | Sprague-Dawley | 175-200g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical | To test the reliability and validity of a novel rat-holding device designed to be used in conjunction with the plantar test apparatus for studying nocifensive behavioral responses in an established model of TMJ pathology |
| Mountziaris et a., 2012 | Males | Sprague-Dawley | 250-300g | 15 µg CFA in 20 µl | Meal pattern | Clinical + Biological + Histological | To investigate the in vivo therapeutic efficacy of an intra-articular controlled release system consisting of biodegradable poly(dl-lactic-co-glycolic acid) (PLGA) microparticles encapsulating anti-inflammatory small interfering RNA (siRNA), together with branched poly(ethylenimine) (PEI) as a transfecting agent, in a model |





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| | | | | | | | of painful TMJ inflammation |
| Wang et al., 2012 | Males | Sprague-Dawley | 200-250g | 25 µg CFA in 50 µl | Head withdrawal | Clinical + Histological | To test the hypothesis that glial activation would regulate the expression of the NMDAR subunit 1 in the trigeminal subnucleus caudalis (Sp5C) induced by TMJ inflammation |
| Wang et al., 2012 | Females | Sprague-Dawley | 180-200g | 0.5mg MIA in 50µl | Head withdrawal | Clinical + Histological | To evaluate whether MIA injection into the upper compartment of the TMJ can be used to create a comprehensive TMJOA model |
| Ivanusic et al., 2011 | Males | Sprague-Dawley | 100-300g | 1 µg CFA in 2 µl | Head withdrawal (Semmes-Weinstein aesthesiometer) | Clinical + Biological | To determine whether peripheral NMDA receptors are involved in inflammation-induced mechanical hypersensitivity of the TMJ |
| Burgos et al., 2010 | Males | Wistar | 200-250g | 50µl of Formalin 2.5% | Behaviour: orofacial rubbing | Clinical + Biological | To evaluate the systemic effect of the cannabinoid agonist WIN 55,212-2 (WIN) and two antagonists (SR141716A and SR144528) on 2 different models of inflammatory orofacial pain ; To compare the effect of WIN on orofacial inflammatory pain with its effect in a model of spinal inflammatory pain (the paw formalin test) ; To compare the antinociceptive effectiveness of WIN with other well-known analgesic drugs such as morphine, indomethacin, and ketamine |
| Kramer et al., 2010 | Males | Sprague-Dawley | 250g | 250 µg CFA in 50 µl | Meal pattern | Clinical + Biological | To show that a meal pattern can measure a persistent increase in TMJ nociception |
| Nicoll et al., 2010 | Males | Wistar | 397+/- 93g | Mouth openning: 2N, 1h every day, 7 days | Head withdrawal (Von Frey aesthesiometer) | Clinical + Histological | To develop a model of TMJ pain and to characterize in it the development and temporal response of behavioral hypersensitivity as well as to evaluate if and to what extent a loading protocol is associated with histological changes in the TMJ consistent with osteoarthritic pathology |
| Villa et al., 2010 | Males | Sprague-Dawley | 200-250g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Histological | To characterize the reaction of peripheral nervous system and central nervous system glial cells to the injection of CFA into the TMJ |
| Bonjardim et al., 2009 | Males | Wistar | 200-300g | Mustard oil 1.5, 2.5 or 4.5% | Behaviour: head flinching and orofacial rubbing | Clinical | To improve the previously reported Mustard oil-induced TMJ nociception model by reducing the concentration of the Mustard oil injected and to investigate the potential analgesic activity of systemic dipyrone and tramadol on the nociceptive behavioral responses induced by TMJ application of the Mustard oil |
| Denadai-Souza et al., 2009 | Males | Wistar | 250-300g | 10 µl of Carrageenan 5% | Head withdrawal | Clinical | To determine the time course of some vascular and cellular events (such as vascular permeability, leukocyte influx, TNF-α and IL-1-β production and pain) secondary to carrageenan-induced TMJ arthritis, and the putative involvement of the tachykinin receptor NK1 in the mediation of these events |
| Schütz et al., 2009 | Males + Females | Wistar | NA | 100 µl CFA | Behaviour: orofacial rubbing + Sleep pattern + Meal pattern | Clinical + Biological | To investigate the effect of orofacial pain upon the behavioral and sleep patterns of both sexes and of females in different phases of the estrous cycle |
| Wang et al., 2009 | Males | Sprague-Dawley | 200-300g | 50 µg CFA in 50 µl | Head withdrawal | Clinical + Biological + Histological | To examine the hypothesis that the upregulation of NR1 in Sp5c following inflammation of the unilateral TMJ region would be regulated by IL-6 and NF-κB |
| Lee et al., 2008 | Males | Sprague-Dawley | 220-280g | 50µl of Formalin 5% | Beahviour: orofacial rubbing | Clinical + Biological | To evaluate the hypothesis that central cannabinoid might modulate the antinociceptive roles of mGluRs in formalin-induced TMJ nociception |
| Schütz et al., 2007 | Males | Wistar | NA | 100 µl CFA | Sleep pattern | Clinical | To examine the role of cyclo-oxygenase-2 in that painful condition, while trying to establish the extent to which this enzyme influences the sleep patterns of animals, both when subjected to this experimental model and when merely manipulated |
| Okamoto et al., | Males | Sprague- | 150-250g | 25 µg CFA in 50 µl | Paw withdrawal (Von Frey | Clinical + | To investigate whether persistent TMJ inflammation affects nocifensive behavioral responses evoked by |

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| 2006 | | Dawley | | | aesthesiometer) | Biological | formalin injection into the hindpaw or withdrawal thresholds of mechanical stimulation to the hindpaw |
| Rodrigues et al. 2006 | Males | Wistar | 200-300g | Carrageenan (100 µg in 15 µl) | Behaviour: head flinching and orofacial rubbing | Clinical + Biological | To show that administration of indomethacin before the initiation of inflammation would diminish the TMJ hyperalgesia |
| Ahn et al. 2005 | Males | Sprague-Dawley | 220-280g | 50µl of Formalin 5% | Behaviour: orofacial rubbing | Clinical + Biological | To investigate the effects of intraarticular or intracisternal injection of IL-1-β on the formalin induced behavioral responses in the TMJ of freely moving rats |
| Gameiro et al., 2005 | Males | Wistar | 200-230 | Stress exposure + Formalin | Behaviour: head flinching and orofacial rubbing | Clinical + Biological | To evaluate the effects of acute and chronic restraint stress on the nociceptive responses induced by TMJ formalin test |
| Guan e tal., 2005 | Females | Sprague-Dawley | 200-225g | 10 µg CFA in 50 µl | Meal pattern | Clinical + Biological + Histological | To test the effect of estrogen on TMJ swelling and monocytic cell number |
| Kerins et al., 2005 | Males | Sprague-Dawley | 175g | 50 µg CFA in 50 µl | Meal pattern | Clinical | To test the efficacy of COX-2-I anti-inflammatory drug Rofecoxib on TMJ inflammation |
| Okamoto et al., 2005 | Males | Sprague-Dawley | 150-200g | 25 µg CFA in 50 µl | Behaviour: orofacial rubbing | Clinical | To evaluate the effect of local administration of the 5HT2AR antagonist, ketanserin, or the 5HT1AR antagonist, propranolol, on the orofacial nocifensive behavior evoked by the injection of formalin |
| Oliveira et al., 2005 | Males | Wistar | 150-250g | Carrageenan (100 µg in 25 µl) | Behaviour: head flinching and orofacial rubbing | Clinical | To investigate whether activation of P2X receptors located within the TMJ region induces nociception |
| Takeda et al., 2005 | Males | Wistar | 280 - 320g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | Clinical + Biological + Electrophysiological | To investigate whether the local release of substance P from the trigeminal root ganglion neurons innervating TMJ modulates the excitability of β-trigeminal root ganglion neurons innervating the facial skin via the paracrine mechanism |
| Kerins et al., 2004 | Males | Sprague-Dawley | NA | 15 µg CFA in 50 µl | Meal pattern | Clinical | To confirm previous findings and extend them by using Rofecoxib, a selective cyclooxygenase-2 inhibitor/ |
| Gameiro et al., 2003 | Males | Wistar | 200-300g | 50µl of Formalin 1.5% | Behaviour: head flinching and orofacial rubbing | Clinical | To evaluate the effect of acute and chronic administration of ethanol and ethanol withdrawal on the pain |
| Hartwig et al., 2003 | Males | Sprague-Dawley | 300-400g | Mustard oil 1, 10 or 20% | Behaviour: chewing + head flinching and orofacial rubbing | Clinical + Biological | To describe and quantify spontaneous noxious stimulus-evoked behaviors in awake rats induced by articular injection of mustard oil |
| Kerins et al., 2003 | Males + Females | Sprague-Dawley | 190g (Males) 230g (Females) | 10 µg CFA in 50 µl | Meal pattern | Clinical + Biological | To further validate our animal model by determining whether aspects of CFA-induced TMJ inflammation/pain are reversed with Ibuprofen treatment |
| Schütz et al., 2003 | Males | Sprague-Dawley | NA | 100 µl CFA | Sleep pattern | Clinical + Biological | To assess an experimental behavioral model of orofacial pain induced by Freund's adjuvant applied into the TMJ while evaluating the sleep pattern and the effect of indomethacin |
| Roveroni et al., 2001 | Males | Wistar | 150-250g | 50µl of Formalin 0.5, 1.5, 2.5 or 5% | Behaviour: chewing + head flinching and orofacial rubbing | Clinical | To apply concentrations of formalin into the TMJ region to develop an experimental behavioral model of TMJ pain and verify if the model proposed is sensitive to morphine and to the hydrophilic lidocaine derivative, QX-314 (2%). |







*Table 2*

| Study | Rat Sex | Rat Breed | Weight | Method of Model Induction | Main Method of | Number of | Painful symptoms |
|---|---|---|---|---|---|---|---|

| | | | | | Nociception Assessment | rats | duration |
|---|---|---|---|---|---|---|---|
| Abdalla et al., 2020 | Males | Wistar | 200-250g | 45µl Fomalin 1.5% | Behaviour: head flinching and orofacial rubbing | 6/group | 14 days |
| de Sousa et al., 2019 | Males | Wistar | 200-250g | 10 µg mBSA in 10 µl | Head withdrawal (Von Frey aesthesiometer) | 6/group | 35 days |
| Ferrara-Jr et al., 2019 | Males | Sprague-Dawley | 250-370g | 100 µg CFA in 100 µl | Head withdrawal (Von Frey aesthesiometer) | 6-11/group | 21 days |
| Garattini et al., 2019 | Males | Wistar | 160-220g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | 6/group | 14 days |
| Sannajust et al., 2019 | Males + Females | Sprague-Dawley | 225-275g (Males) 175-200g (Females) | MIA 16.6mg/ml or 80mg/ml | Head withdrawal (Von Frey aesthesiometer) | 9-12/group | 14 days |
| Scarabelot et al., 2019 | Males | Sprague-Dawley | 250-300g | 25 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | ? (N=52 for 6 groups) | 15 days |
| Zhang et al., 2019 | Females | Sprague-Dawley | 198-271g | 0.5mg MIA in 50µl | Head withdrawal (Von Frey aesthesiometer) | 8/group | 56 days |
| Santos et al., 2018 | Males | Wistar | 250g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | 8/group | 10 days |
| Koop et al., 2017 | Males | Sprague-Dawley | 350-500g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | 5-7/group | 21 days |
| Kartha et al., 2016 | Females | Holtzman | 245 +/- 16.2g | 3,5N mouth openning (1h every day during 7 days) | Head withdrawal (Von Frey aesthesiometer) | 10-12/group | 14 days |
| Scarabelot et al., 2016 | Males | Sprague-Dawley | 250-300g | 25 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | ? (N=35 for 6 groups) | 14 days |
| Magni et al., 2015 | Males | Sprague-Dawley | 200-250g | 60 µg CFA in 60µl | Head withdrawal (Von Frey aesthesiometer) | 4/group | 10 days |
| Cady et al., 2014 | Males | Sprague-Dawley | 300-400g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | 4/group | 14 days |
| Garrett et al., 2012 | Males | Sprague-Dawley | 175-200g | 50 µg CFA in 50 µl | Head withdrawal (Von Frey aesthesiometer) | 6/group | 14 days |





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| Wang et al., 2012 | Females | Sprague-Dawley | 180-200g | 0.5mg MIA in 50µl | Head withdrawal | 3/group | 21 days |
| Kramer et al., 2010 | Males | Sprague-Dawley | 250g | 250 µg CFA in 50 µl | Meal pattern | 13-14/group | 19 days to 42 days |
| Nicoll et al., 2010 | Males | Wistar | 397+/-93g | 2N mouth openning (1h every day during 7 days) | Head withdrawal (Von Frey aesthesiometer) | 4-8/group | 15 days |
| Okamoto et al., 2006 | Males | Sprague-Dawley | 150-250g | 25 µg CFA in 50 µl | Paw withdrawal (Von Frey aesthesiometer) | 12/group | 14 days |

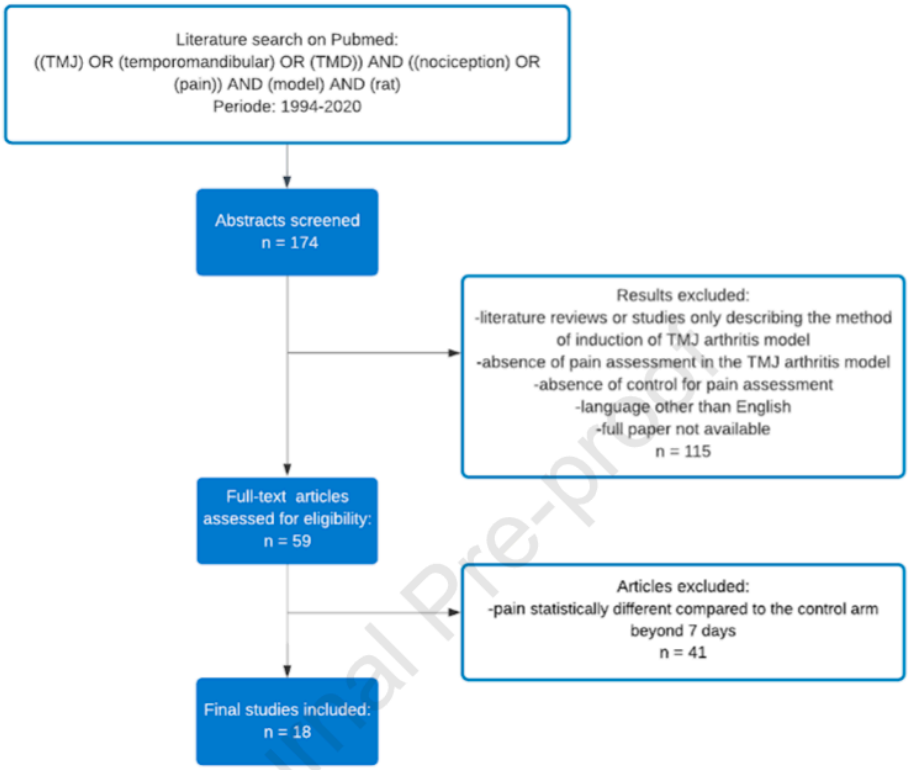
**Tables and Figures:**

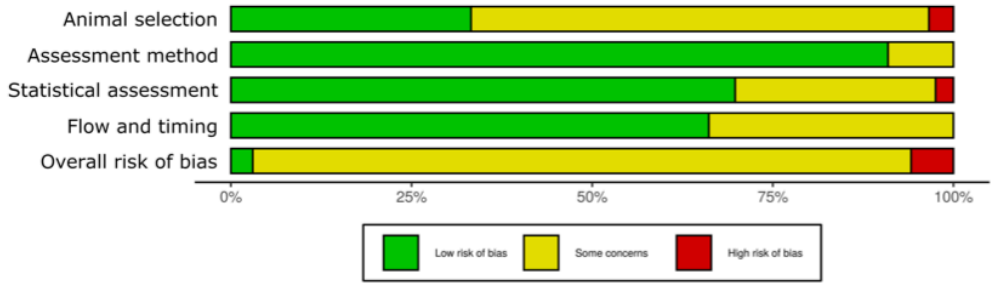
Table 1: Characteristics of eligible studies including a temporomandibular joint osteoarthritis-related pain model.

Table 2: Characteristics of studies including a long-term temporomandibular joint osteoarthritis-related pain model.

Figure 1: Flowchart following PRISMA statement.

Figure 2: Quality assessments of included studies using QUADAS-2 tool. A - Risk of bias summary through a tabular representation resuming the authors' judgments about the risk of each bias item for each included study. B – Risk of bias graph in which items presented as percentages across all included studies.





## 2. Etude comparative de 2 modèles chimiques d'ostéoarthrite temporomandibulaire chez le rat : Collagénase de type II vs Monoacétate iodosodique

INTRODUCTION : L'objectif de cette étude était de comparer deux agents pouvant induire une ostéoarthrite de l'articulation temporomandibulaire (ATM) par induction chimique : l'iodoacétate monosodique (MIA) et la collagénase de type 2 (Col-2). Nous avons cherché à déterminer le meilleur de ces deux agents chimiques dans l'évaluation de systèmes de délivrance de médicaments (DDS).

METHODE : Des rats Wistar mâles ont reçu une injection intra-articulaire de MIA ou de Col-2 et ont été suivis pendant 30 jours. Le seuil de retrait de la tête (HWT), l'évaluation immuno-histologique et la tomographie par émission de positrons (PET) ont été utilisés pour évaluer la pertinence de notre modèle.

RESULTATS : Pour le groupe MIA et le groupe Col-2, la douleur a persisté pendant 30 jours après l'injection. La modification du HWT a montré que la Col-2 déclenchait une action forte au départ qui diminuait ensuite progressivement. Le MIA avait une action constante sur le comportement lié à la douleur. L'histologie de l'ATM a montré une dégradation articulaire progressive au sein des deux groupes.

CONCLUSION : Le MIA et le Col-2 induisaient tous deux une douleur orofaciale par leur action chimique locale sur les ATM. Cependant, sur la base de son effet prolongé et plus soutenu sur l'abaissement des seuils de déclenchement de la douleur, de ses changements histologiques et des résultats de l'imagerie, le MIA nous a semblé être l'agent le plus approprié pour la création d'un modèle d'ostéoarthrite temporomandibulaire cher le rat d'ATM pour l'étude des DDS.

PLOS ONE

RESEARCH ARTICLE

# Comparison of chemical-induced temporomandibular osteoarthritis rat models (monosodium iodoacetate versus collagenase type II) for the study of prolonged drug delivery systems

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## Abstract

### Objective

To compare two agents that can induce a rat model of temporomandibular joint osteoarthritis (TMJOA) by chemical induction: monosodium iodoacetate (MIA) and collagenase type 2 (Col-2). We wished to ascertain the best agent for assessing drug-delivery systems (DDSs).

### Method

Male Wistar rats underwent intra-articular injection with MIA or Col-2. They were manipulated for 30 days. The head withdrawal threshold (HWT), immunohistological assessment, and positron emission tomography (PET) were used to evaluate the relevance of our models.

### Results

For both the MIA and Col-2 groups, pain persisted for 30 days after injection. Change in the HWT showed that Col-2 elicited a strong action initially that decreased progressively. MIA had a constant action upon pain behavior. Histology of TMJ tissue from both groups showed progressive degradation of TMJ components.

### Conclusions

MIA and Col-2 induced orofacial pain by their local chemical action on TMJs. However, based on a prolonged and greater sustained effect on the pain threshold, persistent histological changes, and imaging results, MIA appeared to be more suitable for creation of a rat model of TMJOA for the study of DDSs.

**Competing interests:** The authors have declared that no competing interests exist.

## Introduction

Temporomandibular disorders (TMDs) are myoarthropathies of the manducatory system. They affect ~31% of adults and ~11% of children [1]. TMDs are responsible for most cases of chronic pain in oral and maxillofacial regions. They have a significant impact on healthcare costs and the quality of life (QoL) of patients, and may complicate an existing systemic disease, such as multiple sclerosis [2]. Conventionally, TMDs are divided into two groups according to their manifestation: muscular or articular [3]. Among masticatory muscle disorders are muscle pain, contracture, and hypertrophy. Temporomandibular joint (TMJ) disorders are divided into five separate entities: joint paint, diseases or disorders; congenital disorders; and fractures. The major concern in joint disease TMDs is temporomandibular joint osteoarthritis (TMJOA). TMJOA is a chronic arthritic disease characterized by excessive degradation of bone and cartilage, reduction of the amount of synovial fluid, and persistent synovial inflammation [4, 5]. TMJOA can lead to dysfunctional remodeling of joint components via numerous (and frequently associated) etiologies, including mechanical stress, trauma, dental malocclusion, systemic illness, and hormonal and genetic factors [6, 7].

The symptoms of TMJOA are related to an incessant inflammatory process and osteoarthritis (OA). TMJOA symptoms can vary in intensity over time or can be chronic. Chronic orofacial pain is a key symptom of TMJOA and results from persistent synovitis, muscular spasms, and degenerative arthropathy [6]. Other common symptoms are neck pain, joint noises, and limitation of jaw function [8, 9]. Disease severity ranges from mouth-opening limitation to mandibular ankylosis. Pain symptoms seem to be more common and severe for females than males, probably because of hormonal factors [10, 11].

Treatment of TMJOA requires a complex interdisciplinary approach [12]. The diverse therapeutics applied for the management of this polyfactorial disease can be divided into three categories: non-invasive, minimally invasive, and invasive [3]. Non-invasive techniques include physical therapies, occlusal splints, and pharmacologic agents [13]. Manual therapy, therapeutic exercises, and electrophysiological modalities are validated tools to reduce inflammation, relax muscle tension, and improve jaw mobility. Occlusal splints allow the patient to obtain dental occlusion stability, which minimizes pain in the masticatory muscles and joint by holding the bite in the least joint-traumatizing position [14]. They are also useful in case of parafunctional habits such as bruxism, to avoid tooth attrition, malocclusion, and masticatory muscles fibrosis [15]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed to reduce chronic inflammation but are not without side-effects. Moreover, their short and long-term efficacy are not clearly proven.

Conservative treatment has a real impact [16], but in some cases where noninvasive treatment fails, minimally invasive treatment is applied. Minimally invasive treatment includes intra-articular injections of various substances [e.g., hyaluronic acid (HA)] to enhance the elasticity and viscosity of the synovial fluid and activate tissue repair [17, 18], and NSAIDs and corticosteroids for their anti-inflammatory properties to alleviate pain [19]. Platelet-rich plasma and opioids (e.g., Tramadol™ or morphine) can also be employed. Sometimes, intra-articular injections are combined with arthrocentesis to improve therapeutic efficacy [19].

However, minimally invasive therapies have limitations, such as short-term efficacy because of the rapid clearance and degradation of injectable substances. This phenomenon results in the need for repeated injections, which impacts on QoL (e.g. talking, chewing) and healthcare costs [20], and increases the risk of adverse effects. To overcome these problems, researchers are seeking to develop delivery systems that release drugs over a prolonged period after intra-articular injection [20–22]. Our latest systematic review of the literature on this subject [21] revealed that numerous types of drug-delivery system (DDS) have been investigated, such as poly(lactic-*co*-

glycolic acid)-based biomaterials [23–25], hydrogels [26], microneedle patches [27], nanostructured lipid carriers [28, 29], and poloxamer micelles [30] as carrier molecules, as well as NSAIDs, HA, and analgesic agents as bioactive molecules. There is a growing interest in the development of such therapeutic methods, and they are being tested *in vitro* and *in vivo* [21].

Among animal models developed for preclinical study of the pathology of TMD or the efficacy of therapeutic methods (e.g., DDSs), the rat is used most often because of its cost and size [31]. Numerous methods are available to induce TMJOA-related pain in rats (e.g., chemical, mechanical, surgical). Our systematic review [32] highlighted that, among these methods, intra-articular injection of chemical agents was used most frequently. The two major chemical agents for injection were complete Freund's adjuvant (CFA; which is made from inactivated mycobacteria) and monosodium iodoacetate (MIA). Other drugs or agents can also be applied, including collagenase type 2 (Col-2), formalin, and carrageenan [32]. However, the lack of progressive pathological changes led to several drug-induced models being models of cartilage damage rather than OA and, for others, OA-related symptoms lasted only for a few days, which is not suitable for studying the effect of sustained release of a drug or the prolonged effect of a novel DDS. Therefore, a simple and reproducible animal model of TMJOA that mimics the histopathological changes in cartilage and subchondral bone, as well as clinical symptoms (i.e., pain), over a long period (i.e., months), is needed.

In the present study, we compared MIA and Col-2 for their effects in inducing long-lasting TMJOA symptoms in rats. We did not include CFA because of its origin from a mycobacterium, which means that it must be handled under a biosafety hood to prevent contamination. Col-2 was considered because of its well-known pathological effects on other joints (e.g., knee and hip) [33] and therefore its potentially similar effect on TMJs.

MIA is a selective inhibitor of glyceraldehyde-3-phosphate dehydrogenase. Upon administration by intra-articular injection, it provokes persistent degradation and inflammation by disrupting glycolysis, thereby leading to chondrocyte lysis as well as histological and structural modifications of the TMJ [34]. Hence, MIA is used frequently to establish an OA-like animal model [35, 36] for preclinical research. Several studies have shown the reproducibility and reliability of the MIA-induced TMJOA model in rats [36, 37], which has been applied by other research teams to assess the efficacy of their experimental therapies [38, 39]. *Clostridium histolyticum* Col-2 is a mixture of enzymes containing collagenase, non-specific proteases, and clostripain; it acts mainly on collagen, which is known for its regenerative properties [40]. Intra-articular injection of this product has been shown to induce degradation of cartilage and synovial tissue in induction of an OA model in knee joints [33]. Recently, researchers reported their success in inducing TMJOA using Col-2 [41, 42]. However, our systematic review [32] did not reveal a long-term survey of TMJOA induction using this agent. Therefore, there is a lack of substantial data to validate the reliability of Col-2 for the creation of a TMJOA-related model of prolonged pain in rats. Besides, it is difficult to tell whether MIA or Col-2 is more potent for inducing TMJOA because studies using them have differed with regard to the experimental procedures.

The main objective of this study was to compare MIA and Col-2 to select the one that provides the longest and the most reproducible TMJOA-related pain animal model for the study of prolonged drug release from new DDSs.

## Materials and methods

### Ethical approval of the study protocol

Experimental procedures were carried out at the animal facility within the Platform Ressources Expérimentales of the University of Lille (DHURE, Lille, France). The study protocol was in

compliance with European rules for the protection of animals used for scientific reasons (Directive 2010/63/EU). Surgical procedures were approved (no. 25897) by the local Animal Experimentation Ethics Committee, as well as the French Ministry of Higher Education, Research and Innovation.

## Animals

Forty male Wistar rats (6-weeks-old; 193–267 g) were divided randomly into two groups of 20. Only adult males were used, to avoid biases related to growth and hormonal factors [38]. Animals were housed in a temperature-controlled room (22 ± 1˚C), with a 12-h light–dark cycle. Each rat was placed in an individual cage for ≥7 days. Food and water were available *ad libitum*.

## Chemical induction of TMJOA by intra-articular injection of MIA or Col-2

The injection of MIA or Col-2 into TMJs was applied under an identical protocol. Briefly, under general anesthesia, a solution of the agent was injected into the upper compartment of the left TMJ with a 26-gauge needle following the protocol described by Fuentes and colleagues [43]. To ensure the validity and reliability of this method and to practice our skills, blue dye was used for injection into the TMJs of several rats, and the precision of injection was verified after TMJ dissection.

MIA (0.5 mg; MilliporeSigma, Burlington, MA, USA) was dissolved in 50 μl of physiological (0.9%) saline for injection into the left TMJ of rats in the MIA group. Col-2 (0.2 mg; Millipore-Sigma) was dissolved in 50 μl of 0.9% saline for injection into the left TMJ of rats in the Col-2 group. The concentration and volume of injection used referred to the literature [33–35, 44].

Three time-dependent components (pain assessment, immunohistological changes, and metabolic function) of TMJ tissues were used to compare the efficacy of MIA and Col-2 in inducing TMJOA. The timeline of the study protocol is summarized in Fig 1.

## Nociception assessment by the Von Frey test

The pain experienced by a rat after injection of a chemical agent into the TMJ was measured by using a Von Frey esthesiometer: this is one of the most popular tests for pain evaluation

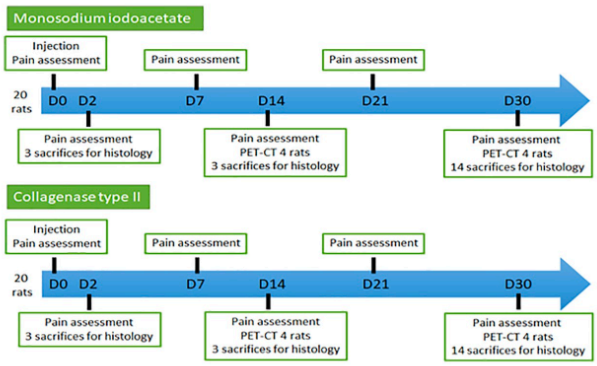


**Fig 1. Study protocol.**

https://doi.org/10.1371/journal.pone.0281135.g001

using animal models. Measurements were made successively before injection of the chemical agent (day 0), then on days 2, 7, 14, 21, and 30 post-injection (Fig 1).

Briefly, before the test, the rat was brought into a quiet room to avoid stress. Then, he was removed from his cage and allowed to move freely for a few minutes. Then, the rat was handled following the guidelines described above for intra-articular injection. A hard-plastic tip was used to stimulate the left-side TMJ. Rat behavior was observed carefully upon increasing the intensity of mechanical stimulation. If head withdrawal or vocalization were observed, the corresponding stimulus intensity (g) was recorded as the head withdrawal threshold (HWT). The HWT was defined as the lowest pressure on the TMJ that induced nociception. Afterwards, the rat was allowed to rest for several minutes. Then, the same manipulation was applied to the right-side TMJ. After each measurement, rats were weighed to monitor their general wellbeing before being returned to their cages.

## Positron emission tomography (PET)

PET was carried out using 2-deoxy-2-[$^{18}$F] fluoro-D-glucose ($^{18}$FDG) as the radiotracer. $^{18}$FDG-PET is useful for TMD evaluation because of its diagnostic performance for OA and its correlation with the therapeutic response [45, 46]. Imaging was undertaken twice on the same five rats from each group on days 14 and 30. $^{18}$FDG (150 μl) was injected into the tail vein with a 28-gauge epicranial needle to produce radioactivity of ~40 mBq. Each step was undertaken applying radioprotection measures. The signal intensity of each group is expressed in standard uptake value (SUV).

## Histology

For each group, on day 2 ($n$ = 3) and day 14 ($n$ = 3), three rats were selected randomly and killed, and on day 30 all remaining rats ($n$ = 14) were killed by intracardiac injection of 0.3 mL of T-61® [embutramide (200 mg/ml), mebenzonium iodide (50 mg/ml), and tetracaine hydrochloride (5 mg/ml); Merck]. Immediately after killing, the whole rat was stored at −80˚C until careful dissection of the entire TMJ sample with surrounding tissues (thickness = 5 mm) (Fig 2). Then, explanted samples were fixed immediately in 10% neutral formalin solution at room temperature. After 24 h, samples were decalcified in a 15% ethylenediaminetetraacetic acid solution (pH 7.2) for 4 days, and then stored in 70% ethanol solution at 4˚C before undertaking the analytical procedures described below. Each sample was placed separately in a cassette for tissue processing, and dehydrated through a graded series of ethanol solutions (70%, 80%, 90%, 95%, and 100% v/v%), and then embedded in paraffin blocks. A series of sections (thickness = 4–5 μm) was cut using a microtome, and then stained with hematoxylin and eosin and toluidine blue (TB) to highlight morphological changes in articular cartilage in the samples. Stained samples of TMJs were imaged at 10× magnification using a scanner (Axio Scan Z1: Zeiss, Jena, Germany). Fig 3 shows an example of histology sections after TB staining, which allowed precise analyses of the different components of the TMJ.

We wished to assess the degree of TMJOA induced by MIA or Col-2 over time. Cartilage degradation was measured by two observers blinded to the study protocol using a modified version of the Mankin Scale [47, 48]. The latter is used widely in preclinical studies on rat models of TMJOA because of its reliability and ease of use [39, 49]. The Mankin Scale is employed to score cellular and background TB staining, chondrocyte arrangement, and the structural condition of cartilage according to damage severity: a score of 0 is given for normal cartilage and a higher score is allotted for more degenerated cartilage. The final score is the sum of the following items: "structure" (0 to 6); "tidemark integrity" (0 to 1); "proteoglycan staining" (0 to 4); and "cellularity" (0 to 3).

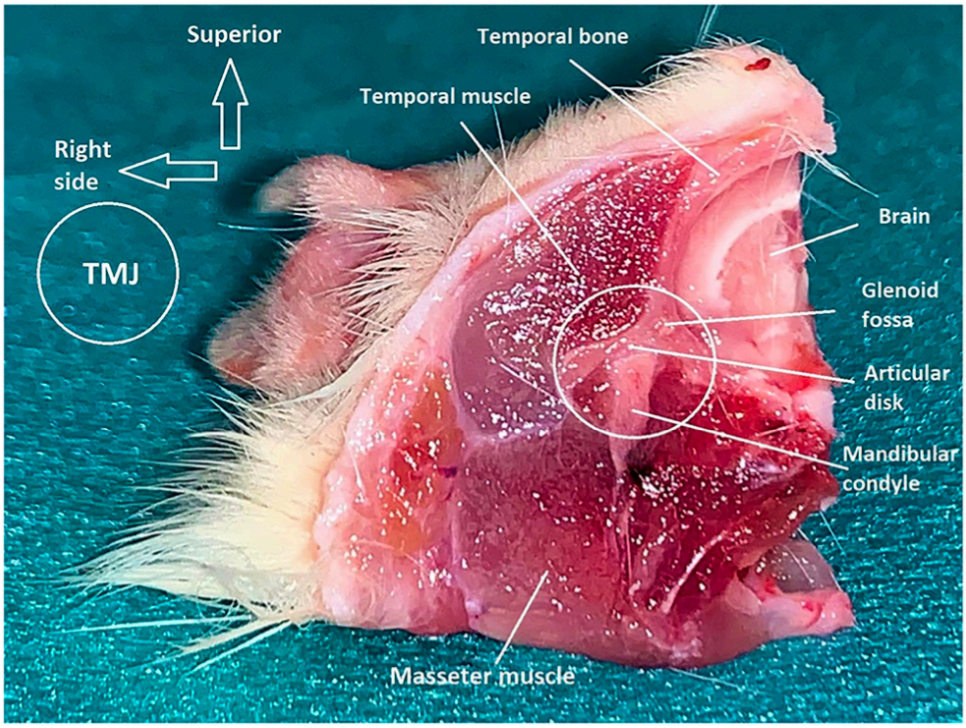


**Fig 2. Entire TMJ sample with description.**

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## Statistical analyses

Datasets were analyzed using frequencies or percentages for categorical variables, and mean ± standard deviation (SD) for quantitative variables. Analyses were based on the non-parametric test, Wilcoxon test, or Mann–Whitney test according to the dependence between samples. $p < 0.05$ was considered significant.

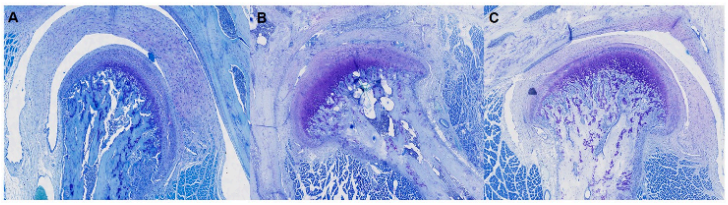


**Fig 3. Examples of histological slides after toluidine blue staining.** A: sample (before injection of MIA or Col-2) without histological and staining modification; B: sample (day 30 Col-2) with a pannus, hypocellularity, and moderate staining reduction; C: sample (day 30 MIA) with hypocellularity, clefts, and severe reduction of proteoglycan staining.

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With regard to nociception assessment, we first considered each group independently. We compared the mean values of the HWT for the left TMJ at different times (day 0 *vs*. day 2, day 0 *vs*. day 7, day 0 *vs*. day 14, day 0 *vs*. day 21, and day 0 *vs*. day 30), and compared the mean values of the HWT for the TMJ on each side of the rat. Then, we compared the mean values of the HWT for the TMJ on the same side in both groups at different times.

With respect to PET assessment, we compared the mean SUV of each group to that of a control group of rats who did not receive an intra-articular injection. Different times (day 14 *vs*. day 30) in the same group, as well as the two groups at the same time, were also compared, respectively.

## Results

### Clinical observation

For both groups, the bodyweight gain of rats was constant during the 30 days of observation.

In the first hours following intra-articular injection of Col-2 into the left-side TMJs, we noticed hemifacial edema in almost all rats. This hemifacial edema stabilized after a few hours and then regressed in the following days. This complication has not been described in the literature.

### Nociception assessment

To understand the relationship between the nociceptive response and histopathological changes, the HWT was measured at different time points after the injection of MIA or Col-2.

**Hyperalgesia of TMJs after MIA injection.** There was no significant difference ($p = 0.461$) between the left TMJ and right TMJ with regard to the HWT before MIA injection (day 0) (Fig 4). The HWT of the MIA-injected side (i.e., the left side) decreased significantly ($p < 0.0001$) 2 days after MIA injection. The HWT decreased significantly until day 30 compared with that at day 0 ($p < 0.0001$). With regard to the right (non-injected) side, a significant decrease in the HWT was found only from day 7 ($p = 0.017$) compared with that at day 0, except for day 21 ($p = 0.323$).

**Hyperalgesia of TMJs after Col-2 injection.** There was no significant difference ($p = 0.277$) between the left TMJ and the right TMJ with regard to the HWT before injection of Col-2 (Fig 5). The HWT of the left-side TMJs decreased significantly ($p < 0.0001$) 2 days after Col-2 injection, and then gradually reached the value seen at day 0, while remaining significantly lower than the baseline value at day 30 ($p = 0.001$). With regard to the (non-injected) right side, a significant decrease in the HWT compared with that at day 0 was observed on days 2 ($p = 0.04$), 7 ($p = 0.036$), 21 ($p = 0.007$), and 30 ($p = 0.036$), but not on day 14 ($p = 0.270$).

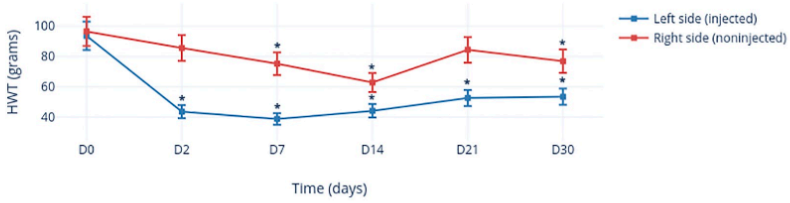


**Fig 4. Change in the HWT of TMJs with/without MIA injection as a function of time.** *Significant values.

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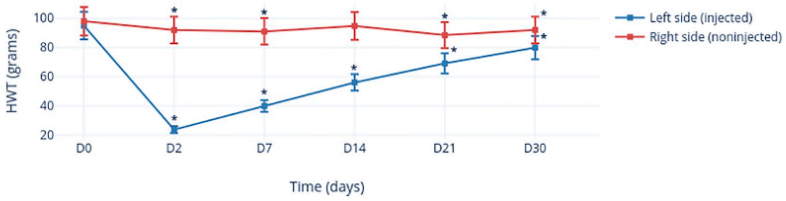


**Fig 5. Change in the HWT of TMJs with/without Col-2 injection as a function of time.** *Significant values.

https://doi.org/10.1371/journal.pone.0281135.g005

**Comparison of the nociceptive response between MIA and Col-2.** A comparison of the pain behavior induced by injecting MIA or Col-2 was based on measurements made on the injected (i.e., the left) side (Fig 6). There was no significant difference ($p = 0.779$) in the baseline value (preinjection) of the HWT between the two groups. On post-injection day 2, the HWT of the Col-2 group (23.961 ± 12.136) was significantly lower ($p < 0.0001$) than that of the MIA group (43.585 ± 17.642). No significant difference was found between the two groups at day 7 ($p = 0.683$). However, from day 14 onwards, the HWT of the MIA group was significantly lower than that of the Col-2 group (day 14: 44.161 ± 17.454 *vs*. 56.091 ±16.503, $p = 0.031$; day 21: 52.645 ± 23.454 *vs*. 69.144 ± 8.704, $p = 0.006$; day 30: 53.460 ± 22.537 *vs*. 79.932 ± 10.746, $p = 0.002$).

## Time-dependent histological changes in TMJs

In some specimens, we observed progressive degradation of the different components of the TMJs described in Fig 3. We documented modification of condyle architecture by the appearance of clefts in different cartilage layers or even of geodes in the case of complete disorganization of condyle architecture. Study of the proteoglycan staining present in the cartilage layers highlights cases of moderate or even severe reduction in the uptake of TB dye, indicating an impoverishment of the cartilage structure. A reduction in the number of chondrocytes, leading to hypocellularity, was also noted.

For both groups we observed a progressive increase in the Mankin Scale score (MIA group: 1.5 ± 1.5 (day 2) < 3.00 ± 1.00 (day 14) < 6.45 ± 2.86 (day 30); Col-2 group: 1.00 ± 0.67 (day 2) < 2.33 ± 1.56 (day 14) < 5.58 ± 3.68 (day 30) (Fig 7). The Mankin Scale scores of the Col-2 group were slightly lower than those of the MIA group. With regard to the right-side TMJs, the profile was irregular in each group.

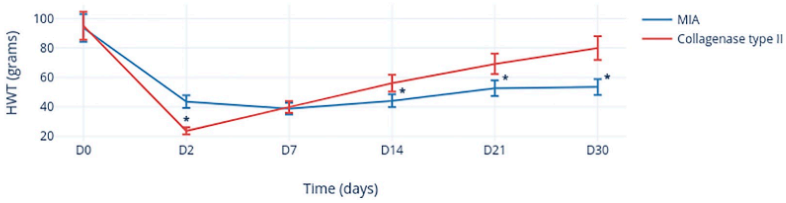


**Fig 6. Change in the HWT after MIA (blue) or Col-2 (red) injection as a function of time.** Values are the mean ± SD and were analyzed using the Mann–Whitney test. *Significant values.

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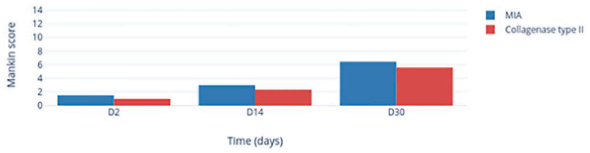


**Fig 7. Change in the Mankin Scale score after MIA (blue) or Col-2 (red) injection as a function of time.**

https://doi.org/10.1371/journal.pone.0281135.g007

## Metabolic changes in TMJs according to PET

For the MIA group, we observed a higher SUV score on the left-side TMJs on day 14 (1.63) and day 30 (1.44) compared with that in the control group (1.30) (Fig 8). For the Col-2 group, a similar SUV score was noted on day 14 (1.27) but a lower SUV score was recorded on day 30 (1.15). PET showed a localized hypersignal at TMJs and in periarticular muscles (Fig 9).

## Discussion

Pain is a predominant clinical feature of OA, and may arise from the soft tissues around the TMJ or the subchondral bone undergoing destruction [6]. Therefore, an accurate animal model of OA should have appropriate nociceptive responses corresponding to its histopathological changes. Here, we created a rat model of TMJOA by injection of MIA or Col-2 into the upper compartment of the TMJ. Intra-articular injection of MIA or Col-2 led to time-dependent pain in rats. The clinical expression of chemically induced pain (represented by evolution of the HWT over time) started 2 days post-injection and lasted at least until day 30 post-injection. Such persistent pain is a necessary criterion for validation of a model in studies of sustained-release DDSs. In this context, MIA and Col-2 met this criterion [21, 32]. Our results for

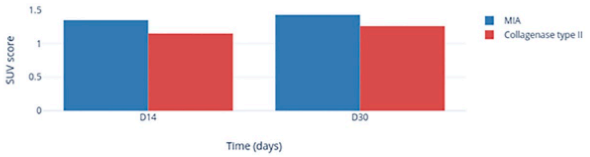


**Fig 8. Change in the FDG level after MIA (blue) or Col-2 (red) injection as a function of time.**

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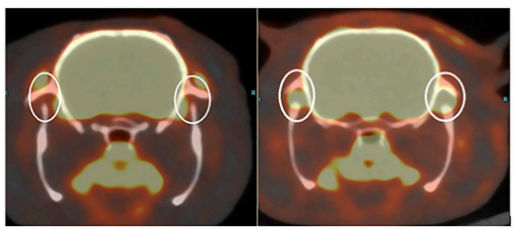

**Fig 9. Comparison between signals at TMJs and periarticular muscles in a non-injected rat (left, SUV score = 1.02) and an MIA-injected rat (right, SUV score = 1.91) at day 14.**

https://doi.org/10.1371/journal.pone.0281135.g009

MIA injection are in accordance with those in recent studies of an MIA-induced model of TMJOA [37], but our results for the creation of a Col-2-induced model of TMJOA are novel.

The TMJs are the two joints that connect the lower jaw to the skull. The TMJs are paired joints that act together. Unilateral injection of chemicals leads to articular degradation *via* mechanical disturbance on the contralateral side [50]. This was demonstrated in our study: for both MIA and Col-2, the pain threshold of the non-injected side decreased gradually from day 7 until day 30. This pattern of pain evolution could be because TMJOA development takes place in two phases. The first is an inflammatory phase in which acute reaction mechanisms are involved and are resolved early (7–10 days); the second is the product of osteoarticular degradation.

Even if MIA and Col-2 are both effective from a clinical viewpoint, comparison between them allows us to highlight differences in the evolution of the pain-triggering threshold and the intensity of the nociception induced by them. The Col-2 group showed a mean HWT value that was significantly lower than that for the MIA group, similar at day 7, and then higher from day 14 to day 30. These data suggest that Col-2 might have a strong effect initially, but this decreases progressively during the first month. In contrast, MIA maintained a constant action on pain behavior in the TMJOA model. This is an important feature to consider in the choice of agent to create a model for testing a DDS, because the model must be as stable as possible over time to ensure that pain relief is from the prolonged action of the drug and not from a decrease in OA [21, 32].

We must not neglect the early strong adverse effects related to intra-articular injection of Col-2, which were not shown by MIA. These features of Col-2 have not been reported previously. Thus, our results could be a warning against the use of Col-2 for this purpose.

Histology revealed remodeling and degradation of articular components after injection of MIA or Col-2 that was aggravated progressively over time and was maximal at the end of the observation period (day 30). The increase in the Mankin Scale score demonstrated the efficacy of the chemical injection to induce a TMJOA model that led to an acute inflammatory (but also a long-lasting) action. The histological signs of prolonged OA corelated closely with the persistence of lower pain thresholds in nociceptive tests. The nociceptive responses of MIA-induced TMJOA corresponded to histopathological changes. Specifically, TMJ hyperalgesia in the first week after MIA injection could mainly be an inflammatory response whereas, 2–4 weeks after MIA injection, hyperalgesia could be corelated to the subsequent destruction of condylar cartilage and erosion of subchondral bone. When synovitis was alleviated and cartilage damage was repaired by fibrous tissue and subchondral bone underwent a sclerotic change, the nociceptive responses correspondingly returned to those observed at baseline. These data were consistent with known clinical features. For example, patients often experience severe pain during the active–destructive phase of TMJOA with synovitis.

PET is used regularly for the clinical diagnosis and follow-up of TMJ diseases [46]. However, use of PET for a TMJOA model is rare. Hence, our protocol for signal quantification was adapted to use the data fully. Use of PET provided interesting data for study of our TMJOA models. PET allows precise visualization of marked inflammatory phenomena in small animals. PET showed a localized hypersignal at the TMJs and in periarticular muscles (Fig 9) that was a reaction to the MIA injection; this effect lasted $\geq$30 days. There was a lack of change in the SUV score in the Col-2 group, a feature which favors use of MIA to establish a TMJOA model.

Therefore, overall, MIA appeared to be more suitable than Col-2 for creating a rat model of TMJOA-related pain for the study of prolonged drug release. The constant character of the pain, which lasted $\geq$30 days, is the principal reason for this conclusion, because pain is the principal symptom of TMJOA.

This is the first study proposing a protocol for creation of a rat model for study of a DDS [32]. The long-term (30-day) nature of our study allowed creation of a reproducible model for study of DDSs. Another advantage was the combination of clinical, histological, immunohistochemical, and PET data to study different aspects (pain and OA) of this complex disease, which can be used to verify the global action of a DDS.

Our study had one main limitation, related mostly to technical difficulties encountered during animal manipulation. The SD values of the HWT in the MIA group tended to be greater than those for the Col-2 group. This finding may imply a progressive improvement of our skills in handling of the rats. Therefore, a longer learning curve for animal handlers than expected may be necessary.

## Conclusions

MIA and Col-2 induced orofacial pain by their local chemical action on TMJs. However, based on its prolonged and greater sustained effect on the pain threshold, persistent histological changes and imaging results, MIA appeared to be more suitable than Col-2 for creation of a rat model of TMJOA. Use of MIA produces a long-lasting TMJOA-related pain animal model for the study of prolonged drug release from new DDSs. This model will also be valuable for other teams because of its ease of use and reproducibility.

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**Funding acquisition:** Romain Nicot.

**Methodology:** Feng Chai, Romain Nicot.

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**Writing – original draft:** Florent Barry.

**Writing – review & editing:** Feng Chai, Henry Chijcheapaza-Flores, Maria José Garcia-Fernandez, Nicolas Blanchemain, Romain Nicot.

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## V. Évaluation de comparateurs dans un modèle d'ostéoarthrite temporomandibulaire chez le rat

INTRODUCTION : Les dysfonctions temporomandibulaires sont un groupe de pathologies complexes, responsables de douleurs orofaciales chroniques. Parmi les traitements actuels, l'injection intramusculaire de toxine botulique A a déjà montré son efficacité dans certaines dysfonctions temporomandibulaires comme les douleurs myofasciales masticatoires. Son injection intra-articulaire a été principalement étudiée dans l'arthrose du genou ou de l'épaule et a montré des résultats prometteurs avec une réduction de la douleur mais reste controversée. Le but de notre étude était d'évaluer l'effet de l'injection intra-articulaire temporomandibulaire de toxine botulique A (incobotulinumtoxinA) sur la douleur en utilisant le modèle d'ostéoarthrite temporomandibulaire chez le rat.

METHODE : Un modèle d'ostéoarthrite temporomandibulaire chez le rat a été utilisé pour comparer l'effet de l'injection intra-articulaire de toxine botulique A (incobotulinumtoxinA), de solution saline (placebo) et d'acide hyaluronique (référence). L'évaluation de la douleur par le test de retrait de la tête, l'analyse histologique quantifiée par l'échelle de *Mankin* et l'imagerie par PET-scan ont été réalisées dans chaque série à différents moments de l'étude jusqu'au jour 30.

RESULTATS : L'injection intra-articulaire de toxine botulique A et d'acide hyaluronique ont montré une diminution significative de la douleur chez les rats par rapport à la solution saline au jour 14. Un effet analgésique statistiquement significatif de la toxine botulique A a été obtenu plus précocement (dès le 7ème jour) et de façon plus prolongée (jusqu'au 21ème jour). Les données histologiques et de l'imagerie montrent une diminution de l'inflammation articulaire dans le groupe toxine botulique A.

CONCLUSION : Cette étude montre un intérêt de l'injection intra-articulaire de toxine botulique A dans un modèle d'arthrose temporomandibulaire chez le rat par rapport à la solution saline.

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# Efficacy of Intra-Articular Injection of Botulinum Toxin Type A (IncobotulinumtoxinA) in Temporomandibular Joint Osteoarthritis: A Three-Arm Controlled Trial in Rats

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# Efficacy of Intra-Articular Injection of Botulinum Toxin Type A (IncobotulinumtoxinA) in Temporomandibular Joint Osteoarthritis: A Three-Arm Controlled Trial in Rats

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**Abstract:** Temporomandibular disorders (TMD) are complex pathologies responsible for chronic orofacial pain. Intramuscular injection of botulinum toxin A (BoNT/A) has shown effectiveness in knee and shoulder osteoarthritis, as well as in some TMDs such as masticatory myofascial pain, but its use remains controversial. This study aimed to evaluate the effect of intra-articular BoNT/A injection in an animal model of temporomandibular joint osteoarthritis. A rat model of temporomandibular osteoarthritis was used to compare the effects of intra-articular injection of BoNT/A, placebo (saline), and hyaluronic acid (HA). Efficacy was compared by pain assessment (head withdrawal test), histological analysis, and imaging performed in each group at different time points until day 30. Compared with the rats receiving placebo, those receiving intra-articular BoNT/A and HA had a significant decrease in pain at day 14. The analgesic effect of BoNT/A was evident as early as day 7, and lasted until day 21. Histological and radiographic analyses showed decrease in joint inflammation in the BoNT/A and HA groups. The osteoarthritis histological score at day 30 was significantly lower in the BoNT/A group than in the other two groups ($p = 0.016$). Intra-articular injection of BoNT/A appeared to reduce pain and inflammation in experimentally induced temporomandibular osteoarthritis in rats.

**Keywords:** osteoarthritis; temporomandibular joint disorders; injection intra-articular; botulinum toxins; type A

**Key Contribution:** Intra-articular injection of BoNT/A appears to reduce pain and inflammation in a rat model of temporomandibular joint osteoarthritis. This treatment could have a place in the management of temporomandibular joint dysfunction in humans.

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## 1. Introduction

Temporomandibular disorders (TMD) are a heterogeneous group of conditions that involve the temporomandibular joint (TMJ) and associated tissues, causing chronic pain, joint noises, limitation of mandibular movement, and impaired quality of life. About 5–12% of the population in industrialized countries are estimated to suffer from TMD [1], with the most common problems being TMJ-related myalgia, arthralgia, and headache, as well as intra-articular pathologies such as disc displacement, degenerative joint disease (osteoarthrosis and osteoarthritis), and subluxation. The management of intra-articular TMD is complex and multidisciplinary. Treatment should be non-invasive in the first instance, with painkillers, physiotherapy, and stress management [2]. Oxygen–ozone therapy is the subject of some studies in the treatment of TMD and shows interesting results [3,4]. For patients not responding to noninvasive measures, pain relief may be obtained with intra-articular injection of hyaluronic acid (HA), platelet-rich plasma (PRP),

or corticosteroids, but the long-term effectiveness is limited, and repeated injections increase the risk of adverse effects [5].

Chronic pain results in peripheral and central sensitization due to excess pain fiber activity, with resultant lowering of the pain threshold. Botulinum toxin type A (BoNT/A) reduces peripheral and central sensitization by decreasing the release of neuropeptides and neurotransmitters from cells or nerve endings [6,7]. Intramuscular injection of BoNT/A has been used for more than 20 years for the treatment of chronic pain and has proven efficacy in TMD with a predominantly muscular component or mixed origin [8,9].

In patients with knee or shoulder osteoarthritis resistant to systemic treatment and intra-articular HA or corticosteroid injections, intra-articular BoNT/A has shown very promising results, reducing pain and improving quality of life without causing major adverse effects [10–18]. In some studies, pain relief after articular BoNT/A lasted up to 8 weeks after the injection [11,13]. Furthermore, in a study of patients with non-neuropathic nociceptive knee pain, Arendt et al. [19] demonstrated a correlation between pain severity and response to BoNT/A. However, some authors are more reserved regarding the efficacy of intra-articular BoNT/A. In a study of 105 patients with knee osteoarthritis, Mendes et al. [20] showed that the pain reduction achieved with intra-articular BoNT/A was not significantly different from that achieved with intra-articular corticosteroid or saline. In a multicenter, double-blind, randomized, placebo-controlled study of 158 patients with knee osteoarthritis, McAlindon et al. [21] found no significant difference in pain reduction between patients treated with intra-articular BoNT/A versus saline. High-powered randomized controlled studies are needed to evaluate the effectiveness of intra-articular BoNT/A in the shoulder, knee, and ankle. The indications must be clearly specified, particularly the osteoarthritis stage, as BoNT/A seems to be more effective in patients with advanced disease and severe pain [22].

There is limited literature on the use of intra-articular BoNT/A in TMJ osteoarthritis (TMJOA) [8]. Two previous studies that evaluated the effect of intra-articular BoNT/A in albumin-induced TMJOA in rats reported a decrease in inflammatory mediators after the injection [23,24]. One of these studies assessed neuropeptide release and pain response (using a behavioral scale) and showed that the peripheral inhibition of release of glutamate, substance P, and calcitonin gene-related peptide (CGRP)—all neuropeptides involved in the pain signal—was responsible for the decrease in arthritis and persistent hypernociception [24]. The first clinical evaluation of intra-articular BoNT/A in humans was conducted by Batifol et al. in 2018 [25]. In this non-controlled study about patients suffering from TMD with severe chronic pain resistant to systemic treatment and intra-articular injections of HA and other drugs, intra-articular BoNT/A (30 IU administered unilaterally or bilaterally) brought about a significant reduction in temporomandibular joint pain up to 3 months after injection, but had no effect on mouth opening and joint noises. In 2022, Sari et al. [26] showed an improvement in pain relief and mouth opening with intra-articular injection of BoNT/A following arthrocentesis in patients with anterior disc displacement.

Given the poor literature but promising data on the effectiveness of BoNT/A in TMJOA, in this three-arm controlled trial we aimed to compare the effect of intra-articular BoNT/A with that of saline (placebo) and HA (as a reference molecule with proven efficacy in the treatment of articular forms of TMD) in a rat model of TMJOA induced by monosodium iodoacetate (MIA) injection.

## 2. Results

### *2.1. Clinical Observation (Body Weight Change)*

All of the rats in the placebo and HA groups gained body weight steadily over time; in contrast, the body weight of the rats in the BoNT/A group slightly decreased initially (between day 2 and day 7) and then gradually increased until day 30 (Figure 1).

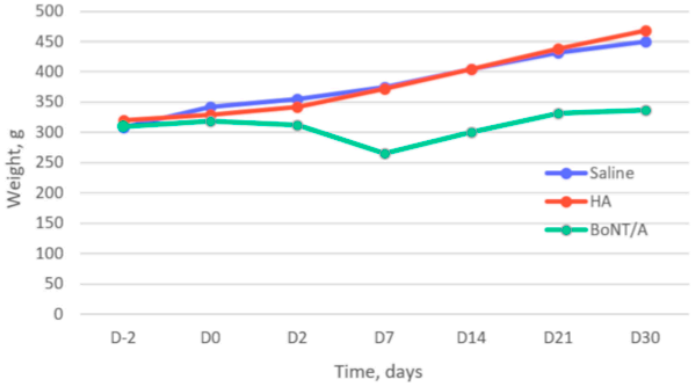


**Figure 1.** Evolution of mean weight of rats over time: before (D-2 and D0) and after (D2 to D30) intra-articular injection of placebo (saline), hyaluronic acid 1% (HA, Ostenil Mini®; TRB Chemedica SAS, Archamps, France), and botulinum toxin A (BoNT/A, incobotulinumtoxinA/Xeomin®; Merz Pharma, Frankfurt am Main, Germany).

### 2.2. Nociception Assessment

Table 1 and Figure 2 show the left TMJ HWT values in the three groups. In each group, the HWT values were significantly lower at day 0 (two days after induction of TMJOA) than at day −2, highlighting that the TMJOA model was well induced. In addition, at day −2 and at day 0 (two days after induction of TMJOA), the HWT values were comparable between the placebo, HA, and BoNT/A groups. At day 2, there were also no significant differences in HWT values between the three groups. At day 7, the mean HWT was significantly lower in the placebo group than in the BoNT/A group (17.56 ± 9.50 vs. 58.06 ± 18.42; $p = 0.05$). At day 14, the mean HWT was significantly lower in the placebo group than in the HA group (16.75 ± 10.29 vs. 65.88 ± 11.62; $p = 0.028$) and the BoNT/A group (16.75 ± 10.29 vs. 66.06 ± 22.53, $p = 0.019$); the difference between the HA group and the BoNT/A group was not statistically significant ($p = 0.422$). At day 21, the mean HWT was significantly lower in the placebo group than in the BoNT/A group (30.91 ± 13.64 vs. 86.01 ± 20.42; $p = 0.048$), but the differences between the placebo group and HA group or between the HA group and BoNT/A group were not statistically significant. At day 30, the mean HWT was comparable between the three groups.

**Table 1.** Left temporomandibular joint head-withdrawal test values (in grams) of rats with temporomandibular joint osteoarthritis at different time points: before (D-2 and D0) and after (D2 to D30) intra-articular injection of placebo (saline), hyaluronic acid 1% (HA, Ostenil Mini®; TRB Chemedica SAS, Archamps, France), and botulinum toxin A (BoNT/A, incobotulinumtoxinA/Xeomin®; Merz Pharma, Frankfurt am Main, Germany). * significant *p* value.

| Left HWT, Grams Mean ± SD | D-2 | D0 | D2 | D7 | D14 | D21 | D30 |
|---|---|---|---|---|---|---|---|
| **Saline** | 91.99 ± 15.03 | 29.81 ± 15.48 | 26.71 ± 9.96 | 17.56 ± 9.50 | 16.75 ± 10.29 | 30.91 ± 13.64 | 47.54 ± 14.19 |
| **HA** | 88.02 ± 9.59 | 44.85 ± 16.68 | 62.96 ± 21.69 | 67.75 ± 15.04 | 65.88 ± 11.62 | 63.03 ± 7.31 | 61.47 ± 6.15 |
| ***p*** | 0.99 | 0.41 | 0.20 | 0.17 | 0.03 * | 0.16 | 0.52 |
| **Saline** | 91.99 ± 15.03 | 29.81 ± 15.48 | 26.71 ± 9.96 | 17.56 ± 9.50 | 16.75 ± 10.29 | 30.91 ± 13.64 | 47.54 ± 14.19 |
| **BoNT/A** | 103.87 ± 11.63 | 45.92 ± 15.39 | 43.73 ± 17.73 | 58.06 ± 18.42 | 66.06 ± 22.53 | 86.01 ± 20.42 | 87.48 ± 11.92 |
| ***p*** | 0.90 | 0.47 | 0.45 | 0.05 * | 0.02 * | 0.05 * | 0.22 |
| **HA** | 88.02 ± 9.59 | 44.85 ± 16.68 | 62.96 ± 21.69 | 67.75 ± 15.04 | 65.88 ± 11.62 | 63.03 ± 7.31 | 61.47 ± 6.15 |
| **BoNT/A** | 103.87 ± 11.63 | 45.92 ± 15.39 | 43.73 ± 17.73 | 58.06 ± 18.42 | 66.06 ± 22.53 | 86.01 ± 20.42 | 87.48 ± 11.92 |
| ***p*** | 0.91 | 0.83 | 0.99 | 0.70 | 0.42 | 0.28 | 0.46 |

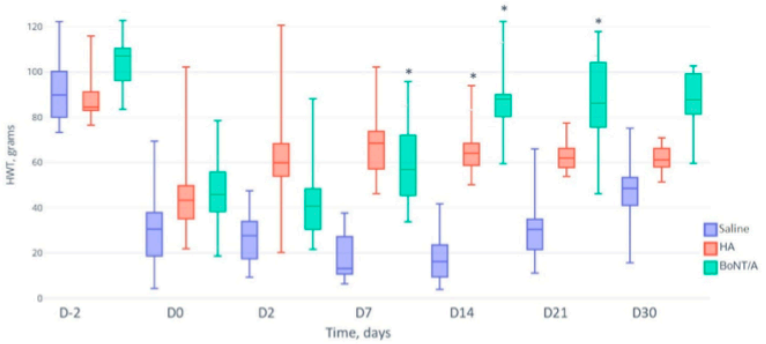


**Figure 2.** Box plot of the left temporomandibular joint head-withdrawal test values (HWT; in grams) of rats with temporomandibular joint osteoarthritis before (D-2 and D0) and after (D2 to D30) intra-articular injection of placebo (saline), hyaluronic acid 1% (HA, Ostenil Mini®; TRB Chemedica SAS, Archamps, France), and botulinum toxin A (BoNT/A, incobotulinumtoxinA/Xeomin®; Merz Pharma, Frankfurt am Main, Germany). * significant *p* value.

### 2.3. Histological Analysis

Figure 3 shows the results of the histological assessment (modified Mankin histological scores) of the left TMJ (with MIA induced osteoarthritis). Changes in the profile of the histological scores over time were different in the three groups. In the placebo group, the Mankin scores were largely similar at different time points: 4.50 ± 0.87 (n = 3) at day 2, 3.83 ± 1.15 (n = 3) at day 14, and 4.15 ± 1.71 (n = 14) at day 30. In the HA group, the Mankin score increased progressively: 2.83 ± 0.76 (n = 3) at day 2, 3.50 ± 0.87 (n = 3) at day 14, and 4.00 ± 2.11 (n = 14) at day 30. Similarly, in the BoNT/A group, the Mankin score increased over time: 1.00 ± 0.87 (n = 3) at day 2, 2.00 ± 0.00 (n = 3) at day 14, and 2.31 ± 1.09 at day 30 (n = 14). The mean Mankin score at day 30 was significantly lower in the BoNT/A group than in the other two groups ($p = 0.016$).

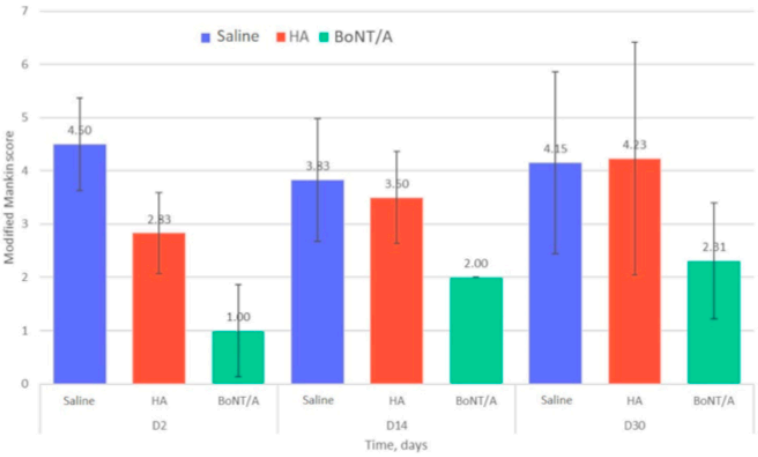


**Figure 3.** Modified Mankin histological scores of rats with temporomandibular joint osteoarthritis at day 2, day 14, and day 30 after intra-articular injection of placebo (saline), hyaluronic acid 1% (HA, Ostenil Mini®; TRB Chemedica SAS, Archamps, France), and botulinum toxin A (BoNT/A, incobotulinumtoxinA/Xeomin®; Merz Pharma, Frankfurt am Main, Germany).

### 2.4. $^{18}$FDG PET Imaging

Table 2 and Figure 4 show the standard uptake value (SUV) on $^{18}$FDG PET at day 30 in each group. SUV expresses the rate of $^{18}$FDG consumption in the area of interest, standardized by the weight of the animal and the dose injected, showing the degree of inflammation and thus osteoarthritis. The median SUV in the left TMJ was comparable between the placebo and HA groups (1.09 [0.95, 1.13] vs. 1.01 [0.86, 1.06], respectively); the median SUV in the BoNT/A group was slightly lower (0.89 [0.88, 0.91]) than the two other groups.

**Table 2.** Standard uptake value measured on $^{18}$FDG positron emission tomography scans performed at day 30 after intra-articular injection of placebo (saline), hyaluronic acid 1% (HA, Ostenil Mini®; TRB Chemedica SAS, Archamps, France), and botulinum toxin A (BoNT/A, incobotulinumtoxinA/Xeomin®; Merz Pharma, Frankfurt am Main, Germany).

| | SUV–Mdn (Q1;Q3) | |
|---|---|---|
| | **Left** | **Right** |
| Saline (n = 5) | 1.09 (0.95;1.13) | 0.9 (0.79;0,90) |
| HA (n = 5) | 1.01 (0.86;1.06) | 1.01 (0.89;1.08) |
| BoNT/A (n = 5) | 0.89 (0.88;0.91) | 0.92 (0.83;0.93) |

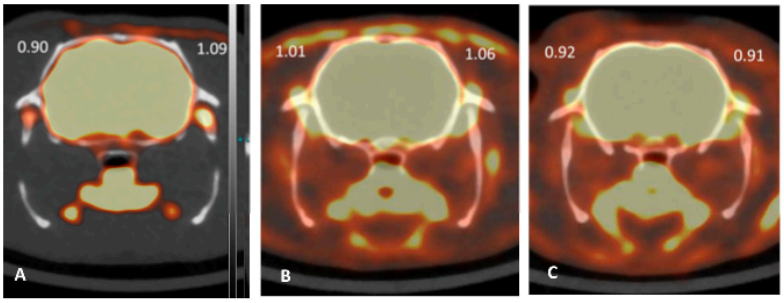


**Figure 4.** Representative $^{18}$FDG PET scans in a rat with temporomandibular joint osteoarthritis at day 30 after intra-articular injection of placebo (saline) (**A**), hyaluronic acid 1% (HA, Ostenil Mini®; TRB Chemedica SAS, Archamps, France) (**B**), and botulinum toxin A (BoNT/A, incobotulinumtoxinA/Xeomin®; Merz Pharma, Frankfurt am Main, Germany) (**C**).

## 3. Discussion

This study evaluates the effect of intra-articular injection of BoNT/A (incobotulinumtoxinA/Xeomin®) versus intra-articular injection of saline or HA in a rat model of TMJOA. The use of the MIA-induced osteoarthritis model, based on the work of Barry et al. [27], allowed for assessment of the effect of these three substances over time. Reduction in TMJOA-related pain after intra-articular injection was similar in the BoNT/A and HA groups at day 14, with both groups having significantly better pain relief than the placebo group. Moreover, the BoNT/A group also had significantly better pain relief than the placebo group at day 7 and day 21.

### 3.1. Generalization

The results of this study are consistent with most previous studies on the subject, finding an effectiveness of BoNT/A in reducing osteoarthritis-related pain [10–18]. Indeed, we showed prolonged improvement in osteoarthritis-related pain from day 7 to day 21 with intra-articular injection of BoNT/A compared with intra-articular injection of the placebo (saline). Some studies also showed prolonged pain relief after intra-articular injection of BoNT/A, lasting up to 8 weeks after the injection [11,13]. Nevertheless, McAlindon

et al. [21] showed contradictory results in human knee osteoarthritis, concluding to no significant difference between the intra-articular injection of BoNT/A and placebo (saline) in reducing the daily average numeric rating scale pain score over 7 days at 8 weeks. Their results are consistent with Mendes et al. [20], who found, in their randomized controlled trial, a higher short-term effectiveness of intra-articular injection of triamcinolone hexacetonide than the intra-articular injection of BoNT/A in reducing pain. It should be noted that the study involved only one dose of botulinum toxin (100 IU in a human knee), which may constitute a bias by underestimating the effectiveness of intra-articular injections of botulinum toxin. Focusing on TMJ, two previous studies on animal models of TMJOA have shown a decrease in pain mediators after intra-articular BoNT/A injection [23,24] and, in addition, Lora et al. [24] demonstrated decrease in pain in behavioral tests. Our results are in line with these previous findings in TMJOA.

Rezasoltani et al. [28] showed that intra-articular BoNT/A was more effective than HA for controlling pain and recovering function in patients in knee osteoarthritis. Conversely, Anil et al. [29] found that intra-articular stromal vascular factor, PRP, and HA were superior to BoNT/A for pain control (assessed by visual analog scale score) and functional outcomes (WOMAC score) in knee osteoarthritis. Our results showed no significant difference in pain improvement in TMJOA treated with intra-articular BoNT/A and HA; however, while pain in the BoNT/A group was significantly lower than in the placebo group at day 7, day 14, and day 21, pain in the HA group was significantly lower than in the placebo group only at day 14. Thus, our results suggest that both BoNT/A and HA can relieve TMJOA-related pain, but the effect of BoNT/A acted earlier and was more prolonged.

### *3.2. Interpretation*

The observed effect of intra-articular BoNT/A on TMJOA-related pain relief is consistent with its known pharmacologic properties. Intra-articular BoNT/A inhibits the release of nociceptive neurotransmitters such as glutamate, substance P, and CGRP, leading to a reduction in pain and inflammation [24]. Glutamate is the main excitatory neurotransmitter in the nervous system of adult mammals and is involved in both pain neurotransmission and central sensitization. Glutamate release has been shown to result in inflammation, pain, and edema [7]. Meanwhile, animal models of adjuvant arthritis and of chronic inflammatory pain have shown marked upregulation of CGRP and mRNA in dorsal root ganglia neurons, as well as elevation of CGRP levels in primary afferent terminals of the spinal dorsal horn [30]. Furthermore, blocking of CGRP has been shown to block behavioral and electrophysiologic signs of enhanced pain in animals with inflammation [31]. In addition, Shi et al. [32] recently reported that the anti-inflammatory effect of BoNT/A in chronic arthritis may also be due to the inhibition of microglial cell activation and the release of microglia-derived tumor necrosis factor α (TNF-α). It is known that microglial cells are activated in chronic pain and release proinflammatory cytokines such as interleukin 6, TNFα, and interleukin 1ß, and thereby cause neuroinflammation. Moreover, P2 × 4 receptors (P2 × 4R) expressed in microglia are involved in neuropathic and inflammatory pain. All of these mechanisms may explain the pain reduction achieved by the intra-articular injection of BoNT/A.

The histological findings in this study offer further evidence in support of the efficacy of BoNT/A in treatment of TMJOA, with the mean modified Mankin score in the BoNT/A group being significantly lower than in the other two groups. The pattern of improvement of osteoarthritis over time was similar in the BoNT/A group and the HA group, but the mean Mankin scores were lower in the BoNT/A group at each time point that those in the HA group. Our findings also suggest that BoNT/A may have an early direct action on the histology, as the modified Mankin score at day 2 was 1.00 ± 0.87 in the BoNT/A group versus 4.50 ± 0.87 in the placebo group. This may be via an effect on the early inflammatory phase of osteoarthritis, with a decrease in the release of inflammatory neuropeptides and the expression of inflammatory cytokines limiting joint degradation [31–33]. Our results

are consistent with the literature, but the mechanism of action of BoNT/A in the TMJ needs to be clarified in future studies.

The PET scan performed at day 30 in each group provided additional supportive information. SUV values were similar in the placebo and HA groups; however, they were slightly lower in the BoNT/A group. Increased $^{18}$FDG tracer uptake was not specific to inflammation, but it could be seen in any area with a significant glycolytic activity, for example, areas with active repair processes. This made interpretation difficult, especially in the HA group. Nevertheless, the lower bilateral SUV values in the BoNT/A group were in favor of a decrease in TMJOA at day 30 and corroborated the clinical and histological findings.

### *3.3. Limitations of the Study*

This study has several limitations. The first was the choice of HA as the reference intra-articular treatment for TMJOA. We chose HA because it is currently the most widely used intra-articular treatment for TMJOA because of its viscosupplementation properties [5]. Other injectable substances such as corticosteroids and PRP are also used. Intra-articular corticosteroids, alone or in combination with other drugs, have not shown better results than intra-articular HA and, moreover, are associated with a risk of condylar resorption [5]. Several studies have shown good results with intra-articular PRP in TMJOA, but the manufacturing protocol is not standardized, and time and special equipment are required to obtain PRP [5,29,34–36]. Second, the weight of the rats in the BoNT/A group initially decreased due to feeding difficulties, probably due to muscle weakness caused by the diffusion of BoNT/A into the masticatory muscles. Change from a hard to a soft diet allowed the rats to eat normally and regain weight. This change in the weight and diet of the rats may have induced stress and behavioral changes, which may have resulted in an underestimation of the pain improvement in the BoNT/A group. In addition, the volume and concentration of the injected BoNT/A was based on the articles by Lora et al. [23,24], and recent studies in humans on the use of high doses of toxin showed a rare occurrence of adverse effects [37,38]. Third, the study sample size of the study was calculated for the statistical analysis of nociception; this sample may have been too small for the statistical analysis of histological and PET imaging data.

## 4. Conclusions

Intra-articular injection of BoNT/A (incobotulinumtoxinA/Xeomin®) appears to be effective for reducing pain in experimentally induced TMJOA in rats. Histological and PET imaging findings support these results. The mean Mankin osteoarthritis histological score at day 30 was significantly lower in the BoNT/A group than in the other two groups.

More high-powered preclinical and clinical studies are needed to determine the place of intra-articular injection of BoNT/A for the treatment of temporomandibular joint osteoarthritis and to draw a firm conclusion.

## 5. Material and Methods

### *5.1. Animals*

Sixty male Wistar rats (6-weeks-old; weight of 250–300 g) were included in this study. The rats were housed in individual cages in a temperature-controlled room (22 °C ± 1 °C) with a 12-h dark–light cycle and allowed for free access to food and water. Manipulations started after ten days of quarantine.

All of the procedures in this study were approved by Ministère de l'enseignement supérieur, de la recherche et de l'innovation (APAFIS#25897, 29/10/2020).

### *5.2. Induction of Temporomandibular Joint Osteoarthritis and Injection Protocol*

The animals were anesthetized by the inhalation of isoflurane mixed with pure oxygen (flow rate: 1.5 L/min) for 2–3 min in a Plexiglas® chamber. TMJOA was induced in the left TMJ of all rats by intra-articular injection of monosodium iodoacetate (MIA) into the

upper compartment in normal saline (0.5 mg/50 µL of saline; Sigma, Saint Louis, MI, USA) [27,39]. The injection protocol was based on the work of Fuentes et al. [40]. Two days after MIA injection, the rats were anesthetized by the same technique and then randomly divided into three groups: 20 rats (the BoNT/A group) received intra-articular injection of 2.5 UI/50 µL BoNT/A (incobotulinumtoxinA; Xeomin®; Merz Pharma, Frankfurt am Main, Germany) in the left and right joint of each rat; 20 rats (the HA group) received intra-articular injection of 50 µL of 1% HA (Ostenil Mini®; TRB Chemedica SAS, Archamps, France) in the left and right joint of each rat; and 20 rats (the placebo group or saline group) received intra-articular injection of 50 µL of 0.9% saline in the left and right joint of each rat. Both Xeomin® and Ostenil® were selected because they are used in clinical practice. In addition, Ostenil® has European certification for use in small joints, including TMJ. Neither Merz Pharma or TRB Chemedica were sponsors of the study.

### *5.3. Nociception Assessment*

The head-withdrawal test (HWT) was used to assess pain. According to the systematic review by Nicot et al. [39], long-term pain related to TMJOA has mostly been assessed by measuring the threshold pressure value (in grams) that triggers a pain response. In this study, a Von Frey aesthesiometer (Figure 5) was applied with gradually increasing pressure to the rat TMJ till head withdrawal, vocalization, or both occurred, indicating nociception; the threshold value was defined as the lowest pressure that induced the response. After each measurement, the rats were weighed (in grams) to monitor their general wellbeing before being returned to their cages.

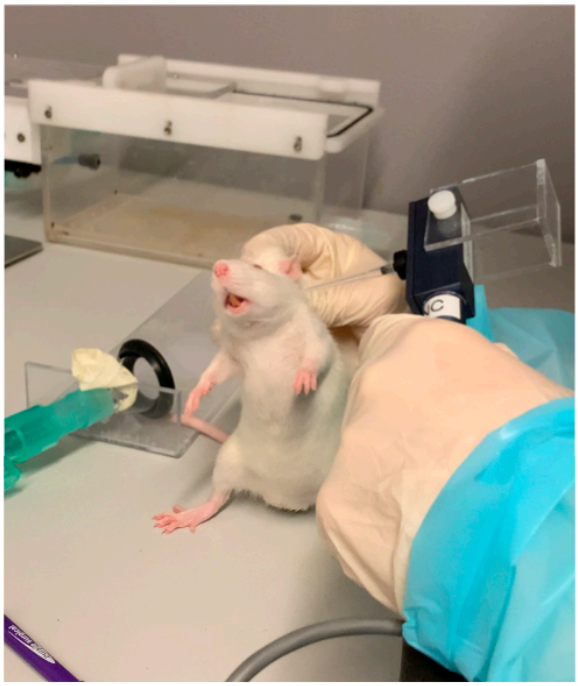

**Figure 5.** Head withdrawal test method: gradually increasing pressure was applied using a Von Frey esthesiometer to the temporomandibular joint area until the animal withdrew its head, vocalized, or both, and the lowest value of pressure (in grams) that induced a response was recorded. The gesture was performed on each temporomandibular joint of each rat.

### *5.4. Histological Analysis*

In each group, randomly selected animals were humanely killed at day 2 (n = 3), day 14 (n = 3), and day 30 (n = 14) by intracardiac injection of 0.2 mL of T61 under isoflurane anesthesia and then immediately stored at −20 °C for at least 48 h. Then, clean-cut samples of approximately 5 mm thickness were obtained from the TMJ area. The samples were first placed in cassettes and fixed in 4% formaldehyde solution for 24 h, and then decalcified by immersion in 15% ethylenediaminetetraacetic acid (EDTA; pH 7.2) solution for 5 days. The prepared samples were stored in 70% ethanol solution at 4 °C until histological processing (paraffin embedding, cutting, and staining) and analysis.

Briefly, the sections were first stained with hematoxylin and eosin (HE) staining to select the slides of interest. The selected slides were thus stained with toluidine blue stain (TB) and examined under the microscope for determining the histological osteoarthritis score using the modified Mankin scale (Table 3). The higher the final score on the Mankin scale, the more advanced the TMJOA stage [41,42].

**Table 3.** Modified Mankin scale for histological scoring of temporomandibular joint osteoarthritis.

| **Structure** | |
|---|---|
| Normal | 0 |
| Surface irregularities | 1 |
| Pannus | 2 |
| Cleft to transitional zone | 3 |
| Cleft to radial zone | 4 |
| Cleft to calcified zone | 5 |
| Complete disorganization | 6 |
| **Tidemark integrity** | |
| Intact | 0 |
| Crossed by blood vessels | 1 |
| **Proteoglycan staining** | |
| Normal | 0 |
| Slight reduction | 1 |
| Moderate reduction | 2 |
| Severe reduction | 3 |
| No dye noted | 4 |
| **Cellularity** | |
| Normal | 0 |
| Diffuse hypercellularity | 1 |
| Cloning | 2 |
| Hypocellularity | 3 |

### *5.5. Positron Emission Tomography (PET) Imaging*

PET with 2-deoxy-2-[18F] fluoro-D-glucose ($^{18}$FDG) was carried out for monitoring the stage of inflammation. $^{18}$FDG radiotracer was used to visualize the areas of high glucose consumption, caused in this case by synovitis and TMJOA bone lesions [43,44]. Imaging was performed at day 30 on five randomly selected rats in each group. The rats were fasted the day before the examination. Intravenous administration of the $^{18}$FDG radiotracer (30–35 MBq) and image acquisition were carried out under general anesthesia. Manipulations were performed in compliance with the rules of human radioprotection [45]. The animals were isolated the first 24 h after radiotracer injection.

*5.6. Full Protocol Frame of the Study*

Figure 6 summarizes the basic frame structure of the full protocol of analysis described below: from TMJOA induction (day −2) to day 30 after therapeutic joint injection.

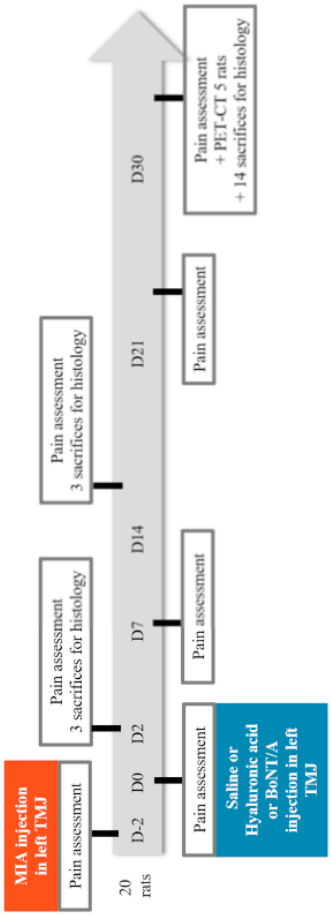


**Figure 6.** Full experimental protocol from temporomandibular joint osteoarthritis induction (day −2) to day 30 after therapeutic joint injection.

*5.7. Statistical Analysis*

The sample size was calculated for three-group one-way analysis of variance (ANOVA) using G*Power 3.1 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany), assuming $\alpha = 0.05$, $\beta = 0.2$, standard deviation (SD) = 10, and effect size = 0.42. The calculated sample size was 19 per group. The final sample size was set at 20 rats per group, keeping in mind potential animal losses and the 3R's rule for experimental procedures in animals [46]. Quantitative variables were expressed as means (±standard deviation) or medians (interquartile range; Q1, Q3), depending on the normality of the distribution. The normality of distributions was assessed using histograms and the Shapiro–Wilk test. Categorical variables were expressed as numbers (percentage). The mean weights and HWT values on day −2 and day 0 were first compared to check the comparability of the three groups. One-way

ANOVA was used to compare the HWT values in the three groups. Levene's test was used to test the homogeneity assumption required by ANOVA. Multiple comparisons within the experimental groups were performed using Tukey's test. One-way ANOVA followed by Dunnett's test was used to compare the placebo group with the experimental groups. Kruskal–Wallis test was used to compare the left TMJ Mankin score at day 30 because the assumptions of one-way ANOVA were not met. All of the statistical analyses were performed using XLSTAT 2022.5.1 (Addinsoft, New York, NY, USA). Statistical significance was at $p < 0.05$.

**Author Contributions:** Conceptualization, R.N.; Methodology, F.C. and R.N.; Formal analysis, M.B. and R.N.; Investigation, M.B., F.B. and R.N.; Writing—original draft, M.B. and R.N.; Writing—review & editing, F.B., M.-J.G.-F., H.C.-F., N.B., F.C. and R.N.; Supervision, N.B., F.C. and R.N.; Funding acquisition, M.-J.G.-F., N.B. and R.N. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The animal study protocol was approved by the Ethics Committee of Ministère de l'enseignement supérieur, de la recherche et de l'innovation (APAFIS#25897, 29/10/2020).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data available on request due to restrictions eg privacy or ethical.

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**Conflicts of Interest:** Author Romain Nicot has been involved as expert consultant in Company Merz Pharma from May 2020 to December 2021 and conducted an educational training program on botulinum toxin injection techniques in validated indications. No conflict of interest with Merz Pharma has to be declared for the present study. The other authors declare no conflict of interest.

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## VI. Conclusions sur les perspectives de prise en charge mini-invasive des dysfonctions temporomandibulaires intra-articulaires

L'utilisation d'un système CHT-PCD sous forme d'hydrogel (CHT/PCDs/PCDi) permet d'associer les caractéristiques de viscoélasticité d'un gel et la libération prolongée locale d'une molécule active tel qu'un AINS et représente donc une option thérapeutique de choix pour le développement d'un hydrogel injectable dans le traitement des principales dysfonctions temporomandibulaires intra-articulaires (déplacements discaux et arthropathies dégénératives).

La formulation et l'optimisation de l'hydrogel sont issues des différents travaux réalisés par l'INSERM U1008 en collaboration avec l'UMET. Le développement d'un hydrogel avec l'ajout d'un principe actif au système CHT-PCD fait l'objet d'un dépôt de brevet européen (Dépôt 22306135.9 du 28 juillet 2022 ; N/Référence : B76705EP / D41400 / EL).

Dans le cadre de l'évaluation de cet hydrogel nu et chargé, nous avons développé un modèle d'ostéoarthrite chimique (induite par l'injection intra-articulaire de MIA) chez le rat avec une évaluation à long terme (30 jours) permettant l'étude d'un système à libération prolongée et testé plusieurs comparateurs (Sérum physiologique, Acide Hyaluronique - Ostenil Mini®, Toxine Botulique A - incobotulinumtoxinA / Xeomin®). Parmi les différents comparateurs, l'acide hyaluronique - Ostenil Mini®, constitue notre molécule de référence et a montré son efficacité dans la prise en charge des douleurs associées à l'ostéoarthrite à J14. La toxine botulique A - incobotulinumtoxinA / Xeomin® présente des résultats intéressants avec une efficacité plus précoce (J7) et plus prolongée (J21).

Parallèlement les tests du système CHT/PCDs/PCDi nu et chargé d'un AINS ont été effectué et mis en perspective par rapport aux différents comparateurs. Un dépôt de brevet européen (Dépôt 22306135.9 du 28 juillet 2022 ; N/Référence : B76705EP / D41400 / EL) a été effectué.

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# Annexes

## Annexe 1.

Gene: **ACTN3** ENSG00000248746 ; Chromosome 11: 66,546,395-66,563,334
Séquence nucléotidique (**exons**)

CCCACGTTGCCCAGCAGGTTGAGCAGCTGGAAGGCGGCCAGCGCCAGCTGCAAGGCCCGGGCCAGGGGTCCCAGC
GGCGGCGGCCCGGGACCGAGCACCAGCACGTAAGCCAGCTCCAGGCCCACGGCCGCGGCCCACAGCGCGGTGAGC
ACGAGAGGCAGCTGCGCGGGCGCCCCGTCCGTGCTCCCAGCCGCCCAGGGCTGCCCCATGGCCTGGACACCCAGC
TGTCGGAGCCGGAGGACTAGGCCGCTTCCGTATTGGGGCGGAGATAGCGGCAGACGGACGTCTGCTACGGCCAAT
AGAAGCTCGGAAGGGGGTGCGGCGAGCGCTCAGACTCTCAGGGAACGGCCGGCCCACGCGGCGAGTTGATCTGGC
CAACGGACCAGCAGTGGAGGCGCTCGGAATCCCGCGGCGCTGGAGCCCGGATAACGCCGCGGAGTGATTGACCCG
CCCACCCTGGAGGCTCCTCCCATCCCGTCGGAGCCCCGCCCGCTGGCGCGGGGGCCAGGAGTGCCCGGGGGATTG
AGTTGCAGCCGGGGTGGTGTCGGGGTTTCCCGGCTCAGCACATTCTCCCCTACTCCCCACAGCCGCCCCGATATT
**AATAGCCCTGCCGCAGCCCATCCAGCTGGCTCAGAGCCGTTCCCCATGCCCGGGTGTCTCCATCACCCCAGAAAA**
**CATGCAAAACAGCTCGGCGCCTCTGTGTTCCTGACTGTGAAATGGGGATAGGGCTCTGTGTCCCACCAGCGAGGT**
**GCTGGAAGAGGCTCACGAGGAGCTCCAGAGGGCAGCCTCTAGAGCGGCCCGCGGCCACTATTATTATCGGCCTTT**
**CTATTGTGGAGAGGAGTAAACAGACTCAGAGAGGTCCCGTGGATTTCTCAAGGTCACACAGCGCCCCAGCAGCGG**
**AGCAGGAATGCAAACCCAGGATCTCCGCCGCGAGATCCACCCCGGCGCCCCCGAGTCCCGCAGTTCCCGGCTTCA**
**GGACAACCCCTTGCCAG**GTCCAACCCGAGCTCCTTCACCCCCATCCGGCCCCTCCCTCCTCCCCCATATTTAGTG
CGAATTCGATCTGGGGCTGGGCTGGGCCCTACTTAATGGGGCCCGGGTGTCCGAGAGC**GTGCCGAGCGGAGCGAA**
**GCCAGGAGCCCGATCGAGATGATGATGGTTATGCAGCCCGAGGGTCTGGGGGCCGGGGAGGGGCGCTTTGCGGGC**
**GGCGGCGGGGGCGGCGAGTACATGGAACAGGAGGAGGACTGGGACCGCGACCTGCTGCTGGACCCGGCCTGGGAG**
**AAGCAGCAGCGGAAA**GTGAGTGCTCGCCCATTTCCTGACAGCAAAACCGAGGCCTGAGAGGCCGGGCTGTTTGTC
CTGGGCATCTCAGCAGTGCAGGGGCAGAGCTTGTCTTTAACCTAGAGTGCTAATCCCCAGCCCAGGCTCTGCCCA
TCAGCTGCCTGCTGTCCCCAGGGCTGGAGGAGAGGGCTGGGAGCCCTCAAGAGGGGTTTAAAATGACAAGAGAGT
TTCTGGGCTCCCCACCCCCACCAGGGTTCATTCCCCTGGGCCCACTGCCCCACTTCTGCTGCTGAAAACAGGCTG
GGCAAACCCCAGGAGATCCAAGTTAGGGGGGGTTCAGAAAGAAGAATCTTCTTCTAGTTAAATATTCCCAGACCC
CACCCTGAAGGCTTGCAGTCAGGCAACTAGTGGACTTGGGAGTGTGGGGGTAGTGAGAAGGGTCCTCTCAGACCC
CACCCCACCTCGTCTCCAGGGTGGGGTCCATAGTCGCTGTATCTCATTTAACAATTGAAGAAACTGAAGCCCAGA
AAGAGCACAGACAGGCCCCACATCACAGACTGTCAGACATCAGGACCCCCGCAACCTGAGGATCTTTACCCTTTT
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GAGGGGAGACCTGGGTCCCCTTACGCTGTAACCTGACTCTCCAGCCAGATGGACACTTAGTGCTGAGGCAGCACC
TCGGGGGCGCCAAAGAGGGGCACAGGGGCAGCTGCTACCCAGGCCCAGCCCGTTGGGCACTCAGGGCCCCTCCCT
CCCTGAGCTATTTTCACTCCCAGACTTTCCCAAGTAGAGGGGGCAGGACACCTGGGTTCCCAAAGGGAAAATGAT
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CTGCTCAGCGTGTGCCAACACTGTATGACTCCAGGAGAGGGCAGGGTACTGGGGTACTCAGTGGAATTTAAATGA
CCTGAGAGTCCCAGAATTTAGAATCAGAGTACTTCTGCTGGGTTAGATGACAGGAGATGACCCCAAGGGCCGAGA
GGCTCCGCTGACTCGGCTGGGGTGCCCCGGGTGACAGGCCTCCCCTTAGGCTCCCCCTGCACCTGTCTCCAAGCA
CGGCTGCAGTCCCAGGTTTGGCAATTTCCCGGTGGTGTCATCCCTGTCCTTGGCTCCAGCTGCCACCTGTTCTGC
AACATCTCCTATCCCTACCCCCACCCACCCGGGCCTCTCCGAGATCCAGGTCTGCGTGCCCCCCATATGACACGT
GCCAGTCTGTAACTTGCCTTTGTCACCTAACAAATTATCAGGAACATCTGTTCATCCCAACTTGCAGTCACCATC
TCATCATCTACTCATTCAATGGCTGGCACAGCATAGAGGATCTGAAAATCAATCAGCATCCCTTGTAGTTGGACA
TTTGGCTCATTTCTTTCTTGTCCAGCGTTGTTTATTGCAAGGTGGTAATAAACATCCTTGTGCTCAGCTTTAGCC
CTGACCCCTCCCTGTTGCTCTATGCTCCCTTTCTCCCCAGTGACTCCTCCATCCAAGGGCTTCCTTCACACAGTC
TTGGAGCAGCACGCTGCCTCCTCCCACCTGATGTCTTACCATGTGCCAGCGCTGTTTCAGGTGCTGGAATTCGGC
AGCAGACAAAATAAAGCCCCTGCCATGGGAGCTTACATTCCAACATTTCTGTCTCATCCATCCAATCCTGCCTTG
TCCCCCACACTGACACCCTTCCTCTCTTGCCCTGATCCCAGTGACTGCGTCCTCACTTCTGCCTTCTGCCACGCC
AAGCCATTCATCTCCACTCTGCAACCAGCATGGACAGTTCTGCCCACCTCCCTCCTGCCTGAGGCTTCCCTGACT
CTCTGTTGCCCTCCGGATCAAATTTTAACTCCCCAGCTTAGCACCCAAGATCACACATGACCTGGCTCCTGCTGC
CCCTCCGGCCTGCCTCTCACCACTTATACCCCAACAATCCCAGCTCCATTCCAGCAGTGCCCAGATCCTTGTAGT
CTGACAAATGTAGCAAGTGTTTCTCACAGCCACCGAGCACTTGCCTGCATGGCAGCCCCTGCCTGAAATGCCCTT
GCCTGCCCTTCCCTTCCTGAATAATTCCTGCCCATCACAGCTCTGATCAAATACTCGCCTTCCCCAAGAGGGGAC
CCTGATCCGCAGCGTCCCTCCCATAAATTCCTCGGCAACCTCTTACAGCGCTTCTCAGACCAAAATGACTCTTGG
ATGGACTGTGAGCTCCCTTAGGGCAGGAACCACATCTCATTAAGAGTCTTGTTTGGGCTGGGAGTGGTGGCTCAT
GCCTGTAATCCCAGCACTTTGGGAGACGGAGGGGGGCGGATCACCTGAGGTTGTAAATTCAAGATCAGCCTGACC
AATATGGAGAAACCCCATCTCTACTAAAAATACAAAATTAGCAGGGCGTGGCGACGCATGCCTGTAATCCCAGCT
ACTTGGGAGGCTGAGGCAGGAGAATCGCTTGAACCAGGGAGGCAGAGGTTGCAGTGAGTAGAGATCGCACCATTG
CACTCCAGTCTGGGCAACAAGCGCGAAACTCTGTCTCCAAAAAAAAAAAAAAAAAAAAAAAAGAGTCATGTTTGGC
ACCACCCAGACCTGCCTATCCCCCTGCCCTTTCCAAACTCAAAGCATCTTAGACTCCAGGGTTGGAGGGAACTGT
GGGAAATCTAACTGCCCTATTCAGTGCTTAGGTTCCCATCAAGTTGTACTCAGTGTCTACTTACATACCTCCAGT
GATGGGAAGCTCACTCCCTTTCCTTAGCAATGACAGCCCCTCAAGTATTTAACGCATCATCTTCCATGTCCAGCT
CCCAGGGCATCTGGCAAGCTGGTCTGAGGAAGGAAAGGTGGCTGAGGGGAAGAATTCCAAATGGCTCCCAGGGCC

ACCTCCCAGGAGTATCTGCTTTGTGGTGTGGGGATGCTCTGAATCCAAGAATCCTGCCAGGCTGGAAGAAGAAAG
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Annexe 2.

Fax émis par : 0155873332 ANSM DP3 15-02-13 18:45 Pg: 1/1

ansm
Agence nationale de sécurité du médicament et des produits de santé

# AUTORISATION D'ESSAI CLINIQUE NE PORTANT PAS SUR UN PRODUIT DE SANTE (ESSAI-HPS)

**Nombre de pages : 1**
(Incluant la page de garde)

## Envoi par Télécopie

**Date :** 15 FEV. 2013

| **Identifiants de l'essai clinique** | | | | | |
|---|---|---|---|---|---|
| Titre | Fondements génétiques du statut musculosquelettique des malocclusions | | | | |
| Promoteur | CHRU de Lille | | | Réf. CPP | |
| Réf. Promoteur | 2012_14 | N° ID RCB | 2012-A01302-41 | Réf. ANSM | B121499-32 |

| **Expéditeur** | **Destinataire** (demandeur : nom / société / tél.) |
|---|---|
| ANSM / Direction Produit NEURHO / Equipe DOLORH | Y.MORICE (secrétariat Fédération de Recherche Clinique)<br>03.20.44.41.45<br>frc@chru-lille.fr |
| Dossier suivi par : Sylvain Guého<br>Tél : 33 (0) 1 55 87 33 41/ Fax : 33 (0) 1 55 87 33 32 | Fax 03.20.44.57.11 |

| **CPP destinataire en copie** | Nord-Ouest IV (Lille) | **Fax** | 03.20.44.68.63 | Code 4 |
|---|---|---|---|---|

Vu le code de la santé publique et notamment ses articles L. 1123-8, R. 1123-32 et vu le dossier de demande d'autorisation d'essai clinique adressé à l'Agence nationale de sécurité du médicament et des produits de santé (ANSM) ;

**L'autorisation mentionnée à l'article L. 1123-8 du code de la santé publique est accordée pour l'essai clinique cité en objet.** Cette autorisation est valable pour toute la durée de l'essai à compter de la date de la présente décision.

Toutefois, conformément à l'article R. 1123-33 du code de la santé publique, la présente autorisation devient caduque si la recherche n'a pas débuté dans un délai d'un an.

Le chef produits ontalgie, rhumatologie, pneumologie, ORL, stomatologie et ophtalmologie
Direction des médicaments en neurologie, psychiatrie, ontalgie, rhumatologie, pneumologie, ORL, ophtalmologie, stupéfiants
**Sylvain GUEHO**

Je vous demande de transmettre toute demande d'informations complémentaires concernant ce dossier par courriel adressé à la boite : **hps-essaiscliniques@ansm.sante.fr**. Je vous précise qu'il vous est possible d'utiliser à cet effet le système de messagerie électronique sécurisée Eudralink. Lors de l'envoi de ces dossiers, je vous demande de veiller à reporter dans l'objet du message les mentions suivantes :

- pour les MS transmises à l'Afssaps pour information : **MSI/ Réf ANSM du dossier**
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Annexe 3.

19/12 2012 14:38 FAX 001

# Comité de Protection des Personnes Nord Ouest IV



Lille, le 14 décembre 2012,

Monsieur FIEVE
Délégation à la recherche
Administration générale
CHRU LILLE

Monsieur le Docteur RAOUL
Service de chirurgie maxillo-faciale et stomatologie
Hôpital Roger Salengro
CHRU LILLE

## COMPTE-RENDU DE DELIBERATION : AVIS

**Référence à rappeler dans toute correspondance : CPP 12/44**
**Intitulé du projet :** ***«Fondements génétiques du statut musculosquelettique des malocclusions»***
**Promoteur** : CHRU LILLE
**Investigateur principal :** Docteur RAOUL
**Référence des documents étudiés:**
**Courrier du promoteur daté du 25/10/12**
**Document additionnel daté du 02/11/12**
**Demande d'autorisation auprès de l'agence française de sécurité sanitaire des produits de santé datée du 02/11/12**
**Attestation d'assurance de la societé SHAM**
**Liste des investigateurs au 11/10/12**
**Protocole version 1 daté du 25/09/12**
**Formulaire de consentement pour un patient majeur non versionné non daté**
**Lettre d'information pour un patient majeur non versionné non daté**
**Lettre d'information pour un patient mineur non versionné non daté**
**Le formulaire de consentement pour un patient mineur non versionné non daté**
**Synopsis version 1 du 25/09/12**
**N° enregistrement** : 2012-A01302-41

**Date de la réunion :** mardi 11 décembre 2012
**Membres présents :**
Mme DE MEDEIROS – Représentant des Infirmiers
Mr S. DUHEM – Représentant des Psychologues
Mme V. BARON – Représentant des travailleurs Sociaux
Mme A. DE BOUVET - Représentant en matière d'Ethique
Mr M. FOULARD - Représentant en matière d'Ethique
Mr R. BEUSCART - Représentant des Personnes Qualifiées en Recherche Biomédicale
Mr THERY - Représentant des Personnes Qualifiées en Recherche Biomédicale
Mme A-F. GERME - Représentant des Personnes Qualifiées en Recherche Biomédicale
Mme Y. VENDEL - Représentant des Personnes Qualifiées en Recherche Biomédicale
Mr F. VASSEUR - Représentant des Personnes Qualifiées en Recherche Biomédicale
Mr N. MESSAADI- Représentant des médecins généralistes
Mme S. COSTA- Représentant des Infirmiers

**Secrétariat :**
E. Broux
Service de Pharmacologie
Faculté de Médecine
Pôle Recherche
1 place de Verdun
59045 LILLE Cedex

Tel : 03 20 44 54 49
Fax : 03 20 44 68 63
Email : cppnordouestiv @univ-lille2.fr

Mme BARON- Représentant des travailleurs sociaux
Mme S. BONTEMPS- Représentant des travailleurs sociaux
Mr G. MARCHAL - Représentant des Associations
Mr P. MACIAG - Représentant des Associations
Mr J-L. CHARDRON - Représentant des Associations
Mr F. MARIE - Représentant des Associations
Mme DANICOURT – Représentant des pharmaciens hospitaliers
Mr P. ODOU - Représentant des pharmaciens hospitaliers
Mme A. LECOCQ – Représentant en matière juridique
Mme L. WILLIATTE - Représentant en matière juridique
Mr P. BARINCOU - Représentant en matière juridique

Monsieur le Directeur, Cher Confrère,

Le Comité de Protection des Personnes Nord Ouest IV, lors de sa réunion du mardi 11 décembre 2012, a pris connaissance des documents concernant l'étude citée en référence

**Le CPP Nord Ouest IV émet un AVIS FAVORABLE à la menée de l'étude avec néanmoins des remarques non bloquantes et non suspensives.**

Dans les critères de non inclusion, il est indispensable de rajouter les femmes enceintes ou allaitantes.

Le Comité signale qu'il faudra revenir vers les patients mineurs pour solliciter à nouveau leur consentement quand ils auront atteint l'âge de 18 ans.

Concernant la note d'information pour patients mineurs, elle démarre par la formule « Madame, Monsieur » qui ne semble pas vraiement adaptée à un patient mineur. L'ensemble de cette note d'information destinée aux mineurs n'est pas acceptable en l'état, c'est un simple copier/coller de la note d'information destinée aux patients adultes, elle mériterait d'être totalement remaniée pour être plus adaptée.

Concernant la note d'information qui s'adresse à un patient majeur, il y a vraisemblablement aussi une erreur de copier/coller puisque cette note d'information est intitulée dans l'encadré « note d'information portant sur la participation d'un mineur à la recherche biomédicale ».

Concernant les formulaires de consentement, il manque la date de signature de l'investigateur.

**Nous vous remercions de nous faire part des réponses à ces remarques non bloquantes et non suspensives**.

Cette étude est conforme aux articles L1121-1 et L1123-7 du code de la santé publique définissant les conditions de validité de la recherche.

*Cet avis a été rendu sans que les membres éventuellement concernés par l'étude aient pris part au vote.*

Je vous prie de croire, Monsieur le Directeur, Cher Confrère, à l'expression de mes meilleures salutations.

Dr Francis VASSEUR
Président du CPP Nord Ouest IV

Mr M. FOULARD – Représentant en matière d'Ethique
Mr X. LABBEE - Représentant en Matière juridique
Mr J-P. JOUET – Expert extérieur

Monsieur le Directeur, Cher Confrère,

Le Comité de Protection des Personnes Nord Ouest IV, lors de sa réunion du mardi 12 février 2013, a pris connaissance des documents concernant l'étude citée en référence.

Le projet avait reçu un avis favorable du CPP Nord Ouest IV lors de sa réunion du mardi 11 décembre 2012. Le CPP avait toutefois émis des remarques non bloquantes et non suspensives.

Les femmes enceintes ou allaitantes ont été ajoutées dans les critères de non d'inclusion. Les notes d'information ont été modifiées selon les demandes du CPP et la lettre d'information destinée aux patients mineurs à été ajoutée. La date de signature de l'investigateur a été ajoutée sur les formulaires de consentement.

Le Comité de Protection des Personnes Nord-Ouest IV vous remercie d'avoir pris en compte les remarques émises.

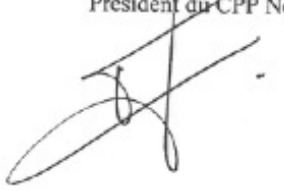
Nous signalons de nouveau qu'il faudra revenir vers les patients mineurs pour solliciter à nouveau leur consentement quand ils auront atteint l'âge de 18 ans. Sauf erreur de notre part, nous n'avons pas retrouvé cette notion dans le protocole.

Cette étude est conforme aux articles L1121-1 et L1123-7 du code de la santé publique définissant les conditions de validité de la recherche.

*Cet avis a été rendu sans que les membres éventuellement concernés par l'étude aient pris part au vote.*

Je vous prie de croire, Monsieur le Directeur, Cher Confrère, à l'expression de mes meilleures salutations.

Docteur Francis VASSEUR
Président du CPP Nord Ouest IV



Monsieur le Directeur, Cher Confrère,

Le Comité de Protection des Personnes Nord Ouest IV, lors de sa réunion du mardi 13 août 2013, a pris connaissance des documents concernant l'étude citée en référence.

Le projet avait reçu un avis favorable du CPP Nord Ouest IV lors de sa réunion du mardi 11 décembre 2012. Le CPP avait toutefois émis des remarques non bloquantes et non suspensives.

Les réponses à ces remarques ont été étudiées par le CPP lors de sa séance du mardi 12 février 2013. Le CPP était satisfait de ces réponses. Il signalait néanmoins qu'il était nécessaire de revenir vers les patients mineurs pour solliciter à nouveau leur consentement lorsqu'ils auront atteint l'âge de 18 ans. Cette correction a été apportée au protocole et une même lettre d'information spécifique pour ces patients a été créée.

**Le Comité de Protection des Personnes Nord-Ouest IV vous remercie d'avoir pris en compte les remarques émises.**

Cette étude est conforme aux articles L1121-1 et L1123-7 du code de la santé publique définissant les conditions de validité de la recherche.

*Cet avis a été rendu sans que les membres éventuellement concernés par l'étude aient pris part au vote.*

Je vous prie de croire, Monsieur le Directeur, Cher Confrère, à l'expression de mes meilleures salutations.

Docteur Francis VASSEUR
Président du CPP Nord Ouest IV

CPP Nord Ouest IV
Le président
Service de pharmacologie
Faculté de Médecine
1 place de Verdun
59045 Lille Cedex

## Annexe 4.

TEMPLE UNIVERSITY®
Office for Human Subjects Protections
Institutional Review Board
Medical Intervention Committees A1 & A2
Social and Behavioral Committee B
Student Faculty Conference Center
3340 N Broad Street - Suite 304
Philadelphia, Pennsylvania 19140
Phone: (215) 707-3390
Fax: (215) 707-8387
e-mail: irb@temple.edu

**Certification of Approval for a Project Involving Human Subjects**

| | |
|---|---|
| Protocol Number: | **13438** |
| PI: | **Sciote, James J.** |
| Approved On: | 04-Oct-2010 |
| Review Date: | 04-Oct-2010 |
| Committee: | A1 - MEDICAL INTERVENTION |
| School/College: | School of Dentistry |
| Department: | ORTHODONTICS-GRADUATE (0723) |
| Project Title: | Musculoskeletal Heritable Infulences on Malocclusion |

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In accordance with the policy of the Department of Health and Human Services on protection of human subjects in research, it is hereby certified that protocol number 13438, having received preliminary review and approval by the department of ORTHODONTICS-GRADUATE (0723) was subsequently reviewed by the Institutional Review Board in its present form and approved on 04-Oct-2010 with respect to the rights and welfare of the subjects involved; appropriateness and adequacy of the methods used to obtain informed consent; and risks to the individual and potential benefits of the project.

In conforming with the criteria set forth in the DHHS regulations for the protection of human research subjects, and in exercise of the power granted to the Committee, and subject to execution of the consent form(s), if required, and such other requirements as the Committee may have ordered, such orders, if any, being stated hereon or appended hereto.

**It is understood that it is the investigator's responsibility to notify the Committee immediately of any untoward results of this study to permit review of the matter. In such case, the investigator should call the IRB at (215) 707-3390.**

***This is the Certificate of Approval. Supplemental documentation will follow under separate cover. Enrollment may not begin until all documents have been reviewed and processed by the IRB and received by the study team.***

Board determined conditions of approval applied to this protocol:

| Name (Fulfilled Date) | Description |
|---|---|
| No Board Conditions | No Board determined conditions of approval assigned to this protocol at this time. |

TEMPLE UNIVERSITY®
Office for Human Subjects Protections
Institutional Review Board
Medical Intervention Committees A1 & A2
Social and Behavioral Committee B
3400 North Broad Street
Philadelphia, Pennsylvania 19140
Phone:215.707.3390 Fax:215.707.8387
e-mail: richard.throm@temple.edu

**MEMORANDUM**

To: **Sciote, James J.**
ORTHODONTICS-GRADUATE (0723)

From: Richard C. Throm
Institutional Review Board

Date: 10-Nov-2010

Re: Expedited Request Status for IRB Protocol:
**13438**: Musculoskeletal Heritable Infulences on Malocclusion

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**This addendum is to be affixed to the IRB Approval Certificate**

45 CFR 46 Protection of Human Subjects.

Expedited review is a type of review that can be conducted by the IRB Chair, other IRB members designated by the Chair, or a subcommittee of the IRB. A major criterion for research that can initially (initial review) reviewed through expedited process is that it must involve no more that minimal risk. The DHHS regulations and FDA regulations define minimal risk to mean that "the probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in the daily life or during performance of routine physical or psychological examinations or tests."

This research protocol was reviewed under the following Expedited Review Category:

**Expedited Category #3:** Prospective collection of biological specimens for research purposes by noninvasive means. Examples: (a) Hair and nail clippings in a nondisfiguring manner; (b) Deciduous teeth at time of exfoliation or if routine care indicates a need for extraction; (c) Permanent teeth if routine care indicates a need for extraction; (d) Excreta and external secretions (including sweat); and (e) uncannulated saliva collected either in an unstimulated fashion or stimulated by chewing gumbase or wax or by applying a dilute citric solution to the tongue; (f) Placenta removal at delivery; (g) Amniotic fluid obtained at the time of rupture of the membrane prior to or during labor; (h) Supra- and subgingival dental plaque and calculus, provided the collection procedure is not more invasive than routine prophylactic scaling or the teeth and the process is accomplished in accordance with accepted prophylactic techniques; (i) Mucosal and skin cells collected by buccal scraping or swab, or mouth washings; (i) Sputum collected after saline mist nebulization.

Annexe 5.

**University of Pittsburgh**
***Institutional Review Board***

3500 Fifth Avenue
Ground Level
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)

**MEMORANDUM**

TO: Alexandre R. Vieira, DDS, MS, PhD

FROM: Christopher Ryan, PhD, Vice Chair

DATE: April 22, 2010

SUBJECT: IRB #0606091: University of Pittsburgh School of Dental Medicine Dental Registry and DNA Repository

Your renewal of the above-referenced proposal has received expedited review and approval by the Institutional Review Board under 45 CFR 46.110 (3,5).

Please include the following information in the upper right-hand corner of all pages of the consent form:

Approval Date: April 21, 2010
Renewal Date: May 12, 2011
University of Pittsburgh
Institutional Review Board
IRB #0606091

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Event Coordinator at 412-383-1504.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month prior** to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

**Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.**

CR:kh

## Annexe 6.


Munich, 18 -21 September 2018

The Scientific Committee of the 24th Congress of the European Association for Cranio Cranio Maxillo Facial held in Munich, September 18-21, 2018
Surgery

CERTIFIES THAT

the following oral paper has been accepted and presented at the Congress:

Introduction To Personalized Medicine In Orthognathic Surgery To Improve Joints Results

Romain Nicot (1), James J. Sciote (2), Alexandre R. Vieira (3), Joël Ferri (1), Gwénaël Raoul (1)

1. University of Lille, Lille, France; 2. Temple University, Philadelphia, United States; 3. University of Pittsburgh, Pittsburgh, United States;

Prof. Klaus-Dietrich Wolff
President EACMFS Munich 2018

Annexe 7.

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

**MINISTERE DE L'ENSEIGNEMENT SUPERIEUR, DE LA RECHERCHE ET DE L'INNOVATION**

Paris, le 29 octobre 2020

**Objet : Notification de décision relative à l'autorisation de projet utilisant des animaux à des fins scientifiques**

**Direction générale de la recherche et de l'innovation**

Service de la performance, du financement et de la contractualisation avec les organismes de recherche

Département des pratiques de recherche réglementées

Cellule Animaux utilisés à des Fins Scientifiques - AFiS -

Affaire suivie par
Véronique Delassault
Responsable administrative de la cellule AFiS

Tél : 01 55 55 97 27
veronique.delassault
@recherche.gouv.fr

autorisation-projet
@recherche.gouv.fr

1 rue Descartes
75231 Paris Cedex 05

En application des dispositions du code rural et de la pêche maritime, notamment ses articles R.214-87 à R.214-126, le projet :

- référencé sous le numéro APAFIS#25897-2020020715156016 v1
- ayant pour titre : Elaboration d'un hydrogel à base de Chitosan avec système de libération prolongée d'anti-inflammatoire non stéroïdien dans la prise en charge des dysfonctions temporomandibulaires
- déposé par l'établissement utilisateur : Plateforme de ressources expérimentales, campus hospitalo-universitaire, Université de Lille - Droit et Santé, numéro d'agrément D5935010, dont le responsable est Monsieur Jean-Christophe CAMART,
- et dont la responsabilité de la mise en œuvre générale du projet et de sa conformité à l'autorisation est assurée par : Madame Feng CHAI, Monsieur Romain NICOT,

est autorisé.

L'autorisation de projet est accordée, sous réserve de la validité de l'agrément de l'établissement utilisateur, pour une durée de 5 ans à compter de la présente notification.

Le projet précité a été évalué sur le plan éthique par le comité d'éthique en expérimentation animale n°075 et a reçu un avis favorable.

Ce projet n'est pas soumis à l'obligation d'une appréciation rétrospective à l'issue de sa réalisation.

Pour la ministre et par délégation
le chef du département
des pratiques de recherche réglementées

Laurent PINON

Monsieur Jean-Christophe CAMART
Plateforme de ressources expérimentales, campus hospitalo-universitaire,
Université de Lille - Droit et Santé