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Evaluation of Salivary Cytokines in Patients with Idiopathic Condylar Resorption of the Temporomandibular Joint

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Evaluation of Salivary Cytokines in Patients with Idiopathic Condylar Resorption of the Temporomandibular Joint

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Oral Biology

By

Linda Hang Phi

2016

ABSTRACT OF THE THESIS

Evaluation of Cytokine Ratios in Patients with Idiopathic Condylar Resorption of the Temporomandibular Joint

by

Linda Hang Phi

Master of Science in Oral Biology

University of California, Los Angeles, 2016

Professor Diana V. Messadi, Chair

The purpose of this study is to evaluate the levels of various cytokines in patients with idiopathic condylar resorption (ICR) of the temporomandibular joint (TMJ). Previous research has assessed the roles of cytokines in serum and synovial fluid of patients with autoimmune diseases resulting in joint degeneration. However, limited studies have assessed saliva as an alternative medium for detection of cytokine levels in ICR. Whole saliva was collected and compared between 10 ICR subjects and 10 control subjects. Using membrane-based protein assay, we found an increase in intensity in the following 12 proinflammatory cytokines: IL-18, IFN-γ, IL-1α, IL-1β, IL-6, TNF-α, IL-17A, IL-17E, GM-CSF, CXCL10, CCL5, and CCL2. A decrease in intensity was seen in the anti-inflammmatory cytokine IL-10. IL-18, IFN-γ, IL-6, TNF-α, IL-17A, IL-17E, and GM-CSF demonstrated statistical significance in our analysis ($p < 0.05$).

The thesis of Linda Hang Phi is approved.

Shen Hu

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Diana V. Messadi, Committee Chair

University of California, Los Angeles

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TABLE OF CONTENTS

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CHAPTER I: INTRODUCTION & BACKGROUND

Idiopathic condylar resorption (ICR) is a term used to describe the phenomenon in which either one or both mandibular condyles display an erosion-like appearance with unknown etiology. Resorption of the condyles is often seen in conjunction with local or systemic pathologies. Local conditions comprise of osteoarthritis, trauma, reactive arthritis, and avascular necrosis[1]. Systemic conditions include rheumatoid arthritis (RA), scleroderma, Sjögren's syndrome, and ankylosing spondylitis[1]. ICR is designated when these pathologies are ruled out in the differential diagnoses.

There is ongoing debate inquiring the role of orthodontics on condylar resorption[2]. Idiopathic condylar resorption is often diagnosed in children, adolescents, and young adults. Many of these patients are either commencing orthodontic treatment, undergoing treatment, or had just completed treatment. Some researchers claim that a dysfunctional remodeling due to orthodontic mechanical stress and unstable occlusion triggers the resorption process. On the other hand, this resorption may also be coincidental, occurring as a result of hormonal changes, parafunction, and other host factors. Despite these differences, researchers can agree that ICR is a multifactorial condition[3-8]; it is possible that ICR can result from all of the previously described factors.

The terms ICR and juvenile idiopathic arthritis (JIA) are often used interchangeably in the health field[7]. However, it is important to remember that ICR describes the state of the mandibular condyles, whereas JIA is an autoimmune, inflammatory joint disease in children. In both disease states, the cause is unknown. JIA may affect one joint or multiple joints[8,9,10]; it may or may not include the mandibular condyle. Although these two disease states may be separate in describing the location of the pathology, it is reasonable to speculate that JIA and ICR may be linked in their pathophysiology and origin. Both diseases are about 9 times more prevalent in

females compared to males[4,11] and primarily affect the joints of adolescent individuals[4,9]. Therefore, it is important to be familiar with JIA in order to attain enhanced insight in the triggering factors for ICR.

Different Types of Juvenile Idiopathic Arthritis

The International League of Associations for Rheumatology (ILAR) has classified JIA into the following 7 different categories[8-10,12]:

1. Systemic arthritis
2. Oligoarthritis
3. Polyarthritis (RF negative)
4. Polyarthritis (RF positive)
5. Psoriatic arthritis
6. Enthesitis-related arthritis
7. Undifferentiated arthritis

A detailed explanation of each JIA category is provided (**Table 1**) along with the exclusion criteria (**Table 2**). Utilizing the criteria, ICR may be closely related with oligoarthritis as it pertains to JIA affecting the TMJ. However, other body joints such as the wrists or knees are seldom affected in ICR. Both ICR and JIA are diagnoses of exclusion; it is important to run laboratory tests to exclude other known autoimmune diseases for proper treatment.

TMJ Anatomy and Composition

Arabshahi et al designates the TMJ as one of the most undertreated and under-diagnosed joints in the human body[13]. The TMJ is a synovial joint situated between the temporal bone and mandible. It is comprised of the mandibular condyle, the temporal bone articulating with the condyle, articular disc, capsule, temporomandibular ligament, stylomandibular ligament,

sphenomandibular ligament, and the lateral pterygoid muscle[13]. It is innervated by the auriculotemporal and masseteric braches of the mandibular branch of the trigeminal nerve.

The TMJ is unique compared to other synovial joints because of its fibrocartilage composition, containing both Type I and Type II collagen[14]. Rather than fibrocartilage, other synovial joints are made of hyaline cartilage containing only Type II collagen[14]. Fibrocartilage is advantageous over hyaline cartilage in the sense that it can tolerate stronger forces during function and occlusion. It is more durable and has a greater ability to repair from damage. However, fibrocartilage may have a greater predisposition to degenerative changes from factors such as sex hormones compared to hyaline cartilage[14]. Three proposed etiologies of TMJ degeneration include altered mechanical loading, female hormonal changes, and extracellular matrix alterations[14]. However, these propositions are speculative, and there is no current evidence proving the causation of TMJ degeneration[14].

CHAPTER II: CLINICAL EVALUATION AND DIAGNOSIS

In general, temporomandibular joint disorders (TMD) have a higher prevalence in women compared to men[4,15]. According to Warren et al, three possible factors contributing to this sex predilection: 1) biological factor, 2) behavioral factor and 3) genetic factor[4]. First, various studies have shown that estrogen and other sex hormones may affect collagen content of the articular disc[4] and synovial tissue hyperplasia[15], as well as their affects on pro-inflammatory cytokines and bone remodeling. Second, women are more likely to report and utilize health services, possibly attributing to the increase in morbidity compared to men. In addition, stress with paranormal functions may also increase propensity of TMD in females, but this area of research has not been fully supported. Lastly, sex chromosomes as it relates to arthritis has been investigated, although more research is underway to support this hypothesis[4].

ICR is generally diagnosed clinically and radiographically, taking into consideration the patient history of occlusion, esthetics, TMJ symptoms, and/or associated pain[1]. In bilateral joint resorption cases, ICR patients commonly present with mandibular retrusion, class II occlusal relationship, and anterior open bite[1]. In unilateral joint resorption cases, ICR patients commonly exhibit a midline shift with a chin deviation to the resorption side, ipsilateral class II occlusal relationship with or without a crossbite, and open bite on the contralateral side[1]. They may also report incidents of clicking, popping, locked jaw, or crepitus; this may be accompanied with pain in one or both TMJs.

Autoimmune or connective tissue diseases should be ruled out with laboratory testing to properly diagnose an individual with ICR. There are currently no tests or criteria for ICR diagnosis. Essentially, ICR is a diagnosis of exclusion when there are no known factors for the TMJ resorption. Various imaging modalities are utilized in order to differentially diagnose ICR from other TMJ derangements.

CHAPTER III: CURRENT IMAGING MODALITIES

Numerous imaging modalities are available in order to visualize both TMJ. The following list describes each imaging technique, as well as their uses in depicting areas of resorption: lateral cephalometric radiography, lateral cephalometric tomography, panoramic radiography, cone-beam tomography (CBCT) scan, ultrasound, and magnetic resonance imaging (MRI).

Lateral cephalometric radiography produces a radiograph of the sagittal plane of the head. Skeletal class II malocclusion, dental class II malocclusion, anterior open bite, high occlusal and mandibular plane angle, and oropharynx narrowing can be detected using lateral cephalometric radiographs[1]. In unilateral ICR cases, the lateral cephalometric radiograph may denote a vertical height discrepancy of the left and right mandibular ramus, as well as an occlusal plane height difference[1]. Lastly, an open bite may be displayed on the unaffected side[1].

Lateral cephalometric tomography will show either normal or large joint space due to the synovial tissue hyperplasia. Loss of cortical bone at the head of the mandibular condyle may be detected with lateral cephalometric tomography[1].

Panoramic radiography, also known as an orthopantomogram (OPG), is a form of tomography where images of multiple planes are taken from ear to ear. It produces a 2-dimentional image of the maxilla, mandible, floor of the orbit, nasal cavity, maxillary sinus, mandibular ramus, inferior border of the mandible, both TMJs, and adjacent structures. After it is confirmed that image distortion due to patient positioning or other variables is eliminated, the mandibular condyles can be evaluated for flattening, resorption, or other anatomical changes[1,13].

Cone-beam computed tomography (CBCT) radiates conical beams from the beam source and takes multiple images at various angles to create cross-sectional images. CBCT creates a 3-dimensional image of the area of interest, giving the radiologist a more accurate

depiction of the mandibular condyles compared to 2-dimensional imaging tools[13]. However, cost and increased radiation compared to conventional modalities must be taken into consideration[16,17].

Ultrasonography formulates real-time images of the area of interest with ultrasound. Advantages to ultrasonography include lower cost compared CBCT and MRI, portability, and lack of ionizing radiation to the patient[13,16].

Magnetic resonance imaging (MRI) utilizes magnetic fields to formulate images of the human anatomy. A decrease in the size of the condyle is seen, with thinning or loss of cortical bone at the condylar head[1]. Anterior disc displacement, either with reduction or without reduction, may be noted on an MRI scan; adverse changes to the articular disc may include deformation or degeneration[1]. Studies have elucidated that MRI is preferred over ultrasound in patients with new-onset JIA due to its higher sensitivity in detecting TMJ changes[13,16,18].

CHAPTER IV: CURRENT TREATMENT MODALITIES

There is presently no standardized treatment protocol for ICR; they include both non-invasive and invasive recommendations. Without an established protocol, treatment options vary widely, comprising of one or more following: physical therapy, oral appliances, systemic medication, arthrocentesis, and surgery.

Physical therapy is often used to increase muscle strength, reduce muscle and joint stiffness, and achieve functional mobility of the TMJ. Warm and moist heat compresses may be included in the therapy to reduce pain[19]. Physical therapy techniques incorporate stress reduction, relaxation, jaw movements, and body posture correction; they also encourage an avoidance of heavy loading exercises, which may worsen the joint pain[19,20].

Oral appliances aid to provide occlusal stability, preventing force overload in one or both joints[19]. As a result, a reduction in muscle pain, joint pain, and muscle soreness may be seen in patients using splints[20]. One study by Shen et al reported pain and clicking in a 24-year-old female patient with a Class II division 1 malocclusion, overjet, and deep bite[21]. One month after splint therapy delivery, the pain and clicking were reportedly decreased; fourteen months after delivery, new bone growth and bone cortication have occurred[21]. One theory explaining condylysis is avascular necrosis, leading to condylar height loss and malocclusion[15]. By revascularizing the bone, there is potential for bone growth and cortication.

Systemic medications are often used to moderate and reduce inflammation. Some medications used to treat ICR and other autoimmune diseases that may result in condylar degeneration include hyaluronic acid[19], corticosteroids[18-20], non-biologic disease-modifying anti-rheumatic drugs (DMARDs)[22], and biologic DMARDs[22]. Non-biologic DMARDs include methotrexate, leflunomide, and sulfasalazine[22]. Biologic DMARDs include TNF-α inhibitors, etanercept, adalimumab, infliximab, abatacept, canakinumab, rilonacept, IL-6 inhibitors,

rituximab, and tofacitinib[22]. Often times, muscle relaxants are prescribed to reduce muscle contraction, subsequent soreness and pain[19].

Arthrocentesis of the TMJ involves the insertion of two cannulas or needles in the joint space, accompanied by lavage. Saline is generally used as the irrigating solution. One needle is used to wash the joint space while the second needle is used to drain synovial fluid and saline[16,23-25]. The technique for arthrocentesis varies among clinicians, and can include either the single-needle or double-needle technique for joint derangements[23]. Additionally, it can be performed with our without ultrasound[25], although more research needs to be performed for ultrasound-guided arthrocentesis. Arthrocentesis has been shown to decrease pain while increasing maximum opening and lateral excursive movement range[19].

Surgery is used to recontour or reconstruct the TMJ into a functional state. Surgeries vary depending on the severity and salvageability of the joint. They can include osseous surgery including arthroplasty, autogenous hemoarthroplasty, alloplastic hemiarthroplasty, osteotomy, osteodistraction, autogenous total joint replacement[19], and total alloplastic temporomandibular joint reconstruction[26-28]. Additionally, soft tissue surgery may include hyperplastic synovial removal, bilaminar tissue removal, disc repositioning, and ligament repair[27,28].

In the case study by Kau et al, a 22-year-old female patient presented with idiopathic condylar resorption, class II skeletal and occlusal relationship, and an anterior open bite[29]. Her treatment included orthodontics combined with a three-piece Le Fort I osteotomy with posterior impaction and expansion. Additionally, she had bilateral inverted L-osteotomy for ramus lengthening. Postoperative relapse of idiopathic condylar resorption has been controversial, with some cases showing a cessation of condylar resorption and retention[29] while others experienced relapse[30].

All in all, a multidisciplinary approach is recommended to treat signs and symptoms of JIA[12], often times combining different modes of treatment with the help of a rheumatologist, social worker, physical therapist, occupational therapy, and surgeon[12].

CHAPTER V: SALIVARY DIAGNOSTICS

There is a shift in analyzing saliva for detection of certain diseases and health statuses[31,32]. Saliva collection is easily available, non-invasive, painless, and less expensive compared to utilizing serum or other bodily samples to identify diseases. Additionally, saliva is easier to transport and handle because it does not clot. Proteins and biomarkers indicative of disease states can cross from serum to saliva[31,32]. Although the proteins are available in much smaller quantity compared to serum, the advancement in technology has allowed researchers to detect these levels. With salivary diagnostics, there is minimal need to prick individuals with needles and resect biopsied tissue[31,32], allowing repeated measurements and sample collection. Lee et al describes saliva as the "mirror of the body," giving insight into not only oral diseases but also systemic diseases[32].

CHAPTER VI: CYTOKINES OF INFLAMMATION

Although various diseases and diagnoses exhibit different initial triggers, bone resorption pathophysiology remains the same for mandibular condylar resorption[33]. According to Wadhwa, TMJ degeneration results from an imbalance of anabolic and catabolic process[14], i.e. osteoblastic and osteoclastic activities. Proinflammatory and anti-inflammatory cytokines are known to play a vital role in cell signaling; a shift to an unbalanced inflammatory state results in condylar resorption with respect to the TMJ. Previous studies have investigated the roles of various proinflammatory and anti-inflammatory cytokines in diseases that result in condylar resorption. The following 13 cytokines will be assessed in this study: IL-18, IFN-γ, IL-1α, IL-1β, IL-6, TNF-α, IL-17A, IL-17E, IL-10, GM-CSF, CXCL10, CCL5, and CCL2.

IL-18

IL-18 is a proinflammatory cytokine belonging to the IL-1 family. Primarily produced by macrophages, it binds to IL-18 receptors activating natural killer (NK) cells[34-40]. Consequently, IFN-γ is produced and activated. In previous studies assessing serum and synovial fluid of patients with JIA, an elevation of IL-18 was found[34,39,41,42]. Kawashima et al reported that in the serum of patients with adult-onset Still's disease, IL-18 level was higher compared to control[35]. In a study by Gracie et al, there were significantly higher levels of IL-18 mRNA and IL-18 protein within the synovial tissues of patients with RA[39]. IL-18 was also found to stimulate osteoclast formation through RANKL upregulation[43], leading to bone resorption; additionally, it has been shown to contribute to cartilage degradation through possible chondrocyte regulation[44].

IFN-γ

NK cells mainly produce IFN-γ, also known as type II interferon, as part of innate immunity. With adaptive immunity, it is primarily produced by CD4 T_H1 and CD8 effector T cells, and it is mediated by IL-12[39]. Additionally, IL-18 induce IFN-γ production[34]. IL-18 forms

a positive feedback loop with T_H1 cells, differentiating T_H0 cells into T_H1 cells that secrete more IFN-γ. During this process, IFN-γ suppresses T_H2 cell differentiation. IFN-γ is pivotal in its role as an acute phase protein, activating macrophages in response to an infection[46,47]. The study by Prahalad et al found an increase in IFN-γ levels in the serum of patients with systemic JIA[48]. The research study by Kawashima et al showed an elevation, although not statistically significant elevation, of IFN-γ in the serum of patients with adult-onset Still's disease[35].

IL-1α

IL-1α belongs to the IL-1 family of cytokines. It is a proinflammatory cytokine capable of promoting fever and inflammation. As acute-phase proteins, IL-1α and TNF-α are both known to activate signaling cascades including p42/p44 mitogen activated protein (MAP) kinase, p54 MAPK, p38 MAPK, NFκB, and β casein kinase[49]. A higher level of IL-1α in synovial fluid compared to serum is correlated with onset of JIA[50].

IL-1β

IL-1β is part of the interleukin-1 cytokine family. Ren et al describes IL-1β as one of the major players of pain, inflammation, and autoimmune diseases[51]. In moderate levels, IL-1β functions as part of homeostasis; however, when IL-1β is overproduced or overexpressed, it becomes a driver of autoimmune diseases such as rheumatoid arthritis and juvenile idiopathic arthritis[51,52]. Caspase 1 is believed to cleave and activate IL-1β, partaking in the immune response cascade[53]. Additionally, IL-1β upregulates mediators of pain, making it a pivotal hyperalgesic agent. Previous studies have shown IL-1β playing a role in mechanical, thermal, and neuropathic pain[51].

IL-6

IL-6 is a proinflammatory cytokine elevated during fever and immune response[41]. It is a member of the IL-6 family of cytokines that binds to the gp130 receptor[54]. During the acute

phase of inflammation, IL-6 and other acute-phase proteins play a role in neutrophil attraction[47,54]. Additionally, studies have shown that IL-6 trans-signaling is needed for recruitment of T cells. Besides recruitment, it is an important cytokine for T cell and B cell differentiation[54]. IL-6 in combination with TGFβ is believed to promote T_H17 cell differentiation, which can stimulate autoimmune diseases such as JIA[54]. Multiple studies have assessed IL-6 levels in serum and synovial fluid of patients with JIA, and they have found an increase in IL-6 levels[35,36,39,48]. Lastly, IL-6 has been found in contributing to osteoclast production leading to bone degradation[55].

TNF-α

TNF-α is a cytokine capable of inducing inflammation and fever. Stimulated by macrophages, it has an essential role in the acute phase of inflammation[47]. However, when TNF-α production and activation is uncontrolled, it can lead to many autoimmune diseases including rheumatoid arthritis and inflammatory bowel disease (IBD). It is also proposed to contribute in the pathogenesis of JIA, although its role is unclear. A study by Pascual et al and a study by Walters et al assessed the serum of patients with RA[56] and JIA[57], respectively. TNF-α inhibitor was determined to be an effective treatment in RA[56]. Similarly, clinical improvement was seen in JIA patients taking TNF-α inhibitor[57].

IL-17A

IL-17A is a proinflammatory cytokine belonging to the IL-17 family. IL-17A can stimulate IL-6 and cyclooxygenase-2 (COX2) expression. Researchers found that T_h17 cells produce IL-17A, assisting in the host defense against bacterial infections from *Staphylococcus aureus, Citrobacter rodentium, and Klebsiella pneumonia* within the first week of infection[58]. It also has a role in protecting the host against fungal infections such as *Pneumocystic carinii* and *Candida albicans*. However, when IL-17A production is dysregulated, chronic inflammation

may occur. This would result in autoimmune diseases such as multiple sclerosis (MS), RA, IBD, and psoriasis, as well as tissue damage[58]. Nistala et al found that IL-17+ T cell expression was significantly higher in joints of patients with JIA compared to serum of both JIA and control patients[59].

IL-17E

IL-17E, also known as IL-25, is also a proinflammatory cytokine belonging to the IL-17 family. It promotes the production of NF-κB and IL-8[60]. As opposed to IL-17A, which is produced by T_h17, T_h2 cells and mast cells produce IL-17E. IL-17E plays a large role in inflammation of the gastrointestinal tract, and chronic inflammation from IL-17E has been implicated to contribute in IBD and other autoimmune diseases[60].

IL-10

IL-10 is an anti-inflammatory cytokine that helps regulate inflammation and the immune response. It works to inhibit T_h1, NK cells, and macrophages[61]. Additionally, IL-10 inhibits MHC Class II and the B7 costimulatory protein, hence preventing the expression of numerous proinflammatory cytokines and chemokines[61]. A review article by Avau et al describes an IL-10 deficiency in serum of patients with JIA[62]. However, the decrease in IL-10 in regulating the inflammatory response may be a contributing factor in many autoimmune diseases, although the exact mechanisms and role is unknown.

GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by a vast array of cells including macrophages, epithelial cells, T cells, mesothelial cells, and fibroblasts[63]. It promotes formation of granulocytes and monocytes from stem cells, making it a vital hematopoietic growth factor and regulator of the immune system. Additionally, GM-CSF induces JAK2 autophosphorylation, initiating the JAK-STAT5 signaling pathway as well as

activating MAPK[63]. GM-CSF overexpression can adversely result in autoimmune diseases such as RA[63]. A research study by Piper et al evaluated serum and synovial fluid of patients with JIA; they found that there was a higher level of GM-CSF in synovial fluid compared to serum of patient with JIA[64]. The authors concluded that T_h17 plasticity may contribute to enhance the levels of GM-CSF-expressing T cells in patients with JIA[64].

CXCL10 (IP-10)

C-X-C motif chemokine 10 (CXCL10), also known as interferon gamma-induced protein 10 (IP-10), is a member of the CXC chemokine family. CD4+ cells, CD8+ cells, NK cells, and NK-T cells produce CXCL10 through IFN-γ influence[45]. CXCL10 works to attract leukocytes, dendritic cells, T cells, and NK cells for cell migration as part of the immune response. However, with T_H1 perpetually producing IFN-γ through the positive feedback loop, CXCL10 levels are magnified. This increase may lead to autoimmune diseases such as RA, psoriatic arthritis, Sjögren syndrome, and systemic lupus erythematosus (SLE)[45].

CCL5 (RANTES)

Chemokine (C-C motif) ligand 5 (CCL5) is also known by the name "regulated on activation, normally T cell expressed and secreted" (RANTES). It is a chemokine that can attract and direct leukocytes into the site of inflammation. Additionally, when CCL5 is high, T cells can release IFN-γ via the tyrosine kinase signaling pathway[65]. Pharaoh et al demonstrated that CCL5 mRNA expression was significantly higher in the joints of patients with JIA compared to serum in three subtypes of JIA: persistent oligoarticular, extended oligoarticular, and polyarticular JIA[65].

CCL2 (MCP-1)

Chemokine (C-C motif) ligand 2 (CCL2) is also known as monocyte chemoattractant protein 1 (MCP1). It is a chemokine that recruits leukocytes and T cells to the site of infection[66].

Whether CCL2 is more effective as a chemoattractant compared to other chemokines is controversial[67]. Anti-CCL2 is proposed as a treatment avenue for some autoimmune diseases such as RA[66]. Additionally, an increase CCL2 was seen in plasma of patients with JIA compared to control[36]. Additionally, higher CCL2 levels were detected in synovial fluid compared to plasma[36].

CHAPTER VII: PURPOSE

The purpose of this study is to evaluate the levels of proinflammatory and anti-inflammatory cytokines in patients with idiopathic condylar resorption (ICR) of the temporomandibular joint (TMJ). The following 13 cytokines were assessed in this study: IL-18, IFN-γ, IL-1α, IL-1β, IL-6, TNF-α, IL-17A, IL-17E, IL-10, GM-CSF, CXCL10, CCL5, and CCL2. Previous research has assessed the roles of cytokines in serum and synovial fluid of patients with autoimmune diseases resulting in joint degeneration. However, limited studies have assessed saliva as an alternative medium for detection of cytokine levels in ICR. In this study, we also wanted to assess saliva as an alternative medium for detection of cytokine levels in ICR.

CHAPTER VIII: METHODOLOGY

Saliva Collection

10 subjects (8 female and 2 male, under the age of 25) with ICR were selected to participate in the study. 10 control subjects, matched by gender and age (8 female and 2 male, under the age of 25), were also selected. Out of the 10 ICR subjects, only 1 reported having been made a splint that was worn only a few times for treatment. The remaining 9 ICR subjects did not have any treatment prior to saliva collection. The 10 control subjects did not report a history of joint pain or any TMJ derangements.

The ICR subjects were verified with Cone Beam CT scans (**Figure 1-10**) and panoramic radiographs (**Figure 11**) of their right and left TMJ. Additionally, they were confirmed clinically with clicking, popping, crepitus, and/or history of jaw locking. A history of TMJ pain was reported in 8 of the 10 subjects. CBCT scans were not taken for the control subjects due to unnecessary radiation.

Prior to saliva collection, subjects were asked to rinse their mouth with water in order to eliminate debris. Unstimulated whole saliva was collected for 3 minutes and immediately placed on ice. The saliva was centrifuged at 10,000 rpm at 4°C for 10 minutes. 25 mL aliquots of the supernatant were collected and stored at -80°C.

Membrane-Based Protein Array

Proteome Profiler Human Cytokine Array (R&D Systems, Minneapolis, MN) was used in the assay. One membrane was used for each sample, resulting in a total of 20 membranes for the 20 samples (10 ICR and 10 control). Each membrane had the capability of reading cytokines in duplicates. We prepared reagents as described in the procedure manual. 2.0 mL of the Array Buffer 4 (block buffer) was pipetted into each well of the 4-well multi-dish. One membrane was placed in each of these wells, and the wells were incubated on a rocking platform shaker for 1

hour. 1 mL of each saliva sample was placed in a tube, adding 0.5 mL of Array Buffer 4 to reach a total of 1.5 mL. 15 mL of reconstituted detection antibody cocktail was added to each tube holding the samples. The tubes incubated for 1 hour. After 1 hour of incubation, Array Buffer 4 was aspirated from the wells of the 4-well multi-dish. The sample mixture containing saliva sample, Array Buffer 4, and reconstituted detection antibody cocktail was added to each of the wells of the 4-well multi-dish. The wells were then incubated overnight on a rocking platform shaker at 2-8°C.

Each membrane was removed and placed into individual containers containing 20mL of 1X wash buffer. Each membrane was washed with 1X wash buffer for 10 minutes on a rocking platform shaker, and the wash was repeated 2 more times for a total of 3 washes. The 4-well multi-dish was washed thoroughly with distilled water and completely dried. Streptavidin-HRP was diluted in Array Buffer 5 to a concentration of 1:2000 as indicated on the vial, and 2.0 mL of this diluted streptavidin-HRP was pipetted into each well of the washed and dried 4-well multi-dish. Each membrane was removed from the wash container, gently draining excess buffer, and added to the 4-well multi-dish. The lid was placed on the dish and incubated for 30 minutes on the rocking platform shaker at room temperature.

The following steps were performed without interruptions. Each membrane was removed from the wash containers, blotting the lower edge of the membrane onto a paper towel. Each membrane was placed on the bottom sheet of the plastic sheet protector, with the identification number facing upwards. 1 mL of the prepared Chemi Reagent Mix was pipetted evenly on the top surface of each membrane, making it a total of 20 mL of prepared Chemi Reagent Mix for 20 membranes. The top sheet plastic sheet protector was placed to cover the membrane. Air bubbles were smoothed out, ensuring that the Chemi Reagent Mix was dispersed evenly to every corner. The membranes were then incubated for 1 minute in the plastic sheet protector. Paper towels

were placed on the top, bottom, and sides of the plastic sheet protector. Excess reagent mix was squeezed out of the plastic sheet protector, draining onto the paper towels. We ensured that no bubbles were present in the plastic sheet protector covering the membranes.

This membrane-plastic sheet-plastic wrap unit was placed in an autoradiography film cassette, with the identification number facing upwards. We exposed the x-ray film at multiple exposure times to assess the best radiographic quality and detection time. The exposure times included 1 minute, 2 minutes, 3.5 minutes, 8 minutes, and 2 hours. We determined that 2-hour exposure produced optimal radiographic image for our analysis (**Figure 13 and 14**).

Analysis of Data

Each spot represented a specific cytokine level based on its coordinates on the membrane (**Figure 12**). Analysis and calculation of cytokine intensity on the membrane-based protein assay was performed with ImageJ (National Institute of Health, USA). Student's *t*-test was used to calculate statistical significance. MedCalc (Belgium) was used to determine the sensitivity, specificity, Receiver Operating Characteristic (ROC) curve, as well as the area under the curve (AUC).

CHAPTER IX: RESULTS

An increase in intensity was observed in IL-18, IFN-γ, IL-1α, IL-1β, IL-6, TNF-α, IL-17A, IL-17E, GM-CSF, CXCL10, CCL5, and CCL2, whereas a decrease in intensity was seen in IL-10. However, only IL-18, IFN-γ, IL-6, TNF-α, IL-17A, IL-17E, and GM-CSF demonstrated statistical significance ($p < 0.05$).

The salivary IL-18 level in ICR patients was much higher (mean ± standard deviation 5305.16 ± 1956.08) compared to control (3666.98 ± 1211.48), resulting in a 1.45 fold change (*p*-value = 0.0371) (**Table 3, Figure 15 and 16)**. IL-18 has a sensitivity of 80% with a specificity of 80% (criterion > 3992.8, AUC = 0.79) (**Table 4 and Figure 17)**.

The IFN-γ level in ICR patients was much higher (3765.52 ± 1643.97) compared to control (2213.46 ± 968.96), resulting in a 1.70 fold change (*p*-value = 0.0192) (**Table 3, Figure 15 and 16**). Similarly to IL-18, IFN-γ sensitivity was calculated to be 80% with a specificity of 80% (criterion > 2595.68, AUC = 0.77) (**Table 4, Figure 17**).

IL-1α level in ICR patients was only slightly higher (5253.14 ± 1840.96) compared to control (5075.80 ± 1502.68), resulting in a 1.03 fold change (*p*-value = 0.8161) (**Table 3, Figure 15 and 16**). The IL-1α sensitivity was lowest compared to the other cytokines, with 40% sensitivity and 80% specificity (criterion > 6115.632, AUC = 0.53) (**Table 4, Figure 17**).

The IL-1β level in ICR patients was moderately higher (3809.94 ± 2403.12) compared to control (2816.48 ± 1153.34), resulting in a 1.35 fold change (*p*-value = 0.2539) (**Table 3, Figure 15 and 16**). Both sensitivity and specificity for IL-1β was higher compared to IL-1α, calculated as 50% sensitivity and 90% specificity for IL-1β (criterion > 4012.184, AUC = 0.61) (**Table 4, Figure 17**).

The IL-6 level in ICR patients was more than twice the IL-6 level in control patients (2101.10 ± 1259.86 and 954.53 ± 433.41, respectively, *p*-value = 0.0140) with a fold change of

2.20, the highest fold change observed compared to other cytokines (**Table 3, Figure 15 and 16**). IL-6 sensitivity was calculated to be 90%, and the IL-6 specificity was determined to be 60% (criterion > 896.473, AUC = 0.82) (**Table 4, Figure 17**).

TNF-α level in ICR patients was only slightly higher (2455.30 ± 1401.04) compared to control (1183.66 ± 765.24), resulting in a 2.07 fold change (*p*-value = 0.0214) (**Table 3, Figure 15 and 16**). The TNF-α sensitivity and specificity was relatively high, with 90% sensitivity and 70% specificity (criterion > 1049.562, AUC = 0.79) (**Table 4, Figure 17**).

The IL-17A level in ICR patients was much higher (1655.03 ± 877.95) compared to control (826.63 ± 459.94), resulting in a 2.00 fold change (*p*-value = 0.0165) (**Table 3, Figure 15 and 16**). Like IL-18 and IFN-γ, IL-17A sensitivity was calculated to be 80% with a specificity of 80% (criterion > 937.55, AUC = 0.81) (**Table 4, Figure 17**).

In comparison to IL-17A level, IL-17E level in ICR patients was not as drastically high (2923.61 ± 1275.65) compared to control (1776.50 ± 585.02), resulting in a 1.65 fold change (*p*-value = 0.0187) (**Table 3, Figure 15 and 16**). IL-17E sensitivity was calculated to be 60% with a specificity of 100% (criterion > 3003.607, AUC = 0.75) (**Table 4, Figure 17**).

IL-10 level was the only cytokine tested showing a decrease in intensity for ICR patients compared to control (1045.76 ± 714.33 and 1397.99 ± 591.48, respectively), resulting in a 0.75 fold change (*p*-value = 0.2453) (**Table 3, Figure 15 and 16**). IL-10 sensitivity was determined to be 50%, and IL-10 specificity was determined to be 90% (criterion < 616.9435, AUC = 0.63) (**Table 4, Figure 17**).

The GM-CSF level in ICR patients was twice the GM-CSF level in control patients (2019.57 ± 1051.43 and 1008.56 ± 416.60, respectively, *p*-value = 0.0112) with a fold change of 2.00 (**Table 3, Figure 15 and 16**). GM-CSF sensitivity was calculated to be 60%, and the GM-

CSF specificity was determined to be 100% (criterion > 1822.942, AUC = 0.77) (**Table 4, Figure 17**).

CXCL10 level in ICR patients was only slightly higher (4023.94 ± 1661.58) compared to control (3451.06 ± 1404.66), resulting in a 1.17 fold change (*p*-value = 0.4160) (**Table 3, Figure 15 and 16**). The CXCL10 sensitivity and specificity was determined to have 50% sensitivity and 80% specificity (criterion > 4596.075, AUC = 0.60) (**Table 4, Figure 17**).

The CCL5 level in ICR patients was moderately higher (1220.85 ± 669.17) compared to control (859.97 ± 695.15), resulting in a 1.42 fold change (*p*-value = 0.2523) (**Table 3, Figure 15 and 16**). CCL5 sensitivity was calculated to be 100% with a specificity of 30%, the lowest specificity among all of the analyzed cytokines (criterion > 209.8515, AUC = 0.64) (**Table 4, Figure 17**).

Like CCL5, CCL2 level in ICR subjects was moderately higher (3047.65 ± 1614.10) compared to control (2125.46 ± 1280.41), resulting in a 1.43 fold change (*p*-value = 0.1740) (**Table 3, Figure 15 and 16**). The CCL2 sensitivity was high and specificity was low, with 100% sensitivity and 40% specificity (criterion > 1255.374, AUC = 0.65) (**Table 4, Figure 17**).

CHAPTER X: DISCUSSION

Our research aimed to assess saliva in ICR patients and control in order to test whether saliva is a good medium to detect pathological changes at the protein level. Previous research studies have utilized synovial fluid and serum to determine proinflammatory and anti-inflammatory cytokine levels in disease patients with autoimmune diseases (e.g. RA, JIA, Still's disease). Few research studies have analyzed saliva for detection of cytokines in these autoimmune diseases. No published studies to this date have analyzed saliva for ICR.

As indicated in the results, an increase in intensity was observed in the following proinflammatory cytokines: IL-18, IFN-γ, IL-1α, IL-1β, IL-6, TNF-α, IL-17A, IL-17E, GM-CSF, CXCL10, CCL5, and CCL2. A decrease in intensity was seen in the anti-inflammatory cytokine IL-10. Only IL-18, IFN-γ, IL-6, TNF-α, IL-17A, IL-17E, and GM-CSF demonstrated statistical significance in our analysis ($p < 0.05$).

IL-6 is a proinflammatory cytokine that is present in most inflammatory diseases; it was determined to have the greatest fold change of 2.20 ($p = 0.0140$) (**Table 3, Figure 15 and 16**) in ICR patients compared to control. With a high sensitivity (90%) and moderately high specificity (60%) (**Table 4, Figure 17**), IL-6 is a reliable cytokine in saliva for detection of ICR.

TNF-α is often the protein targeted for inhibition in treatment of autoimmune diseases such as RA and ICR. Our study showed that TNF-α had the second highest fold change of 2.07 ($p = 0.0214$) (**Table 3, Figure 15 and 16**) in ICR patients compared to control. Like IL-6, TNF-α had both a high sensitivity (80%) and high specificity (70%) (**Table 4, Figure 17**), making it a vital candidate for ICR screening. With this data, TNF-α is a logical candidate for inhibition in patients with ICR.

Both GM-CSF and IL-17A displayed the third highest fold change of 2.00 ($p = 0.0112$ and $p = 0.0165$, respectively) (**Table 3, Figure 15 and 16**). This data supports the previous

studies by Piper et al and Nistala et al, indicating a higher level of GM-CSF and IL-17A in ICR patients compared to control. This data suggests that there may be a shift to T_H17 differentiation and plasticity in ICR pathophysiology. However, statistically significant increases in IL-17E (p = 0.0187) and IFN- γ (p = 0192) (**Table 3, Figure 15 and 16**) indicate that T_H2 and T_H1 differentiation, respectively, cannot be ruled out in ICR pathophysiology.

Recently, researchers have been interested in IL-18 for its possible role in autoimmune diseases such as RA and ICR. Salivary IL-18 one of its product, IFN-γ, exhibited a statistically significant increase in ICR patients compared to control, with similar detected fold changes (fold change = 1.45 p=0.0371, and fold change = 1.70 p = 0.0192, respectively) (**Table 3, Figure 15 and 16**). However, these fold changes were not as high as we had predicted them to be. Nevertheless, both IL-18 and IFN-γ showed high sensitivity (80% for both) and specificity (80% for both) (**Table 4, Figure 17**), potentially making them ideal proinflammatory cytokines for ICR screening and detection.

Surprisingly, IL-1β and IL-10 did not have the statistically significant difference as we had hoped. As a major player of inflammation and pain, IL-1β had a 1.35 fold change (p = 0.2539) (**Table 3, Figure 15 and 16**). It is interesting to note that in our ICR subjects, 80% of individuals reported a history of pain. However, during saliva collection, only 2 individuals reported that they currently experience moderate to severe pain, while the remaining subjects reported mild to no pain. With this, IL-1β may be a candidate biomarker to assess active resorption in ICR with current pain manifestation, but more research needs to be conducted. IL-10 was decreased in ICR patients compared to control (fold change = 0.75), although this decrease was not statistically significant (p = 0.2453) (**Table 3, Figure 15 and 16**). With a sensitivity of 50% (**Table 4, Figure 17**), IL-10 was not easily and reliably detectible in our protein array experiment.

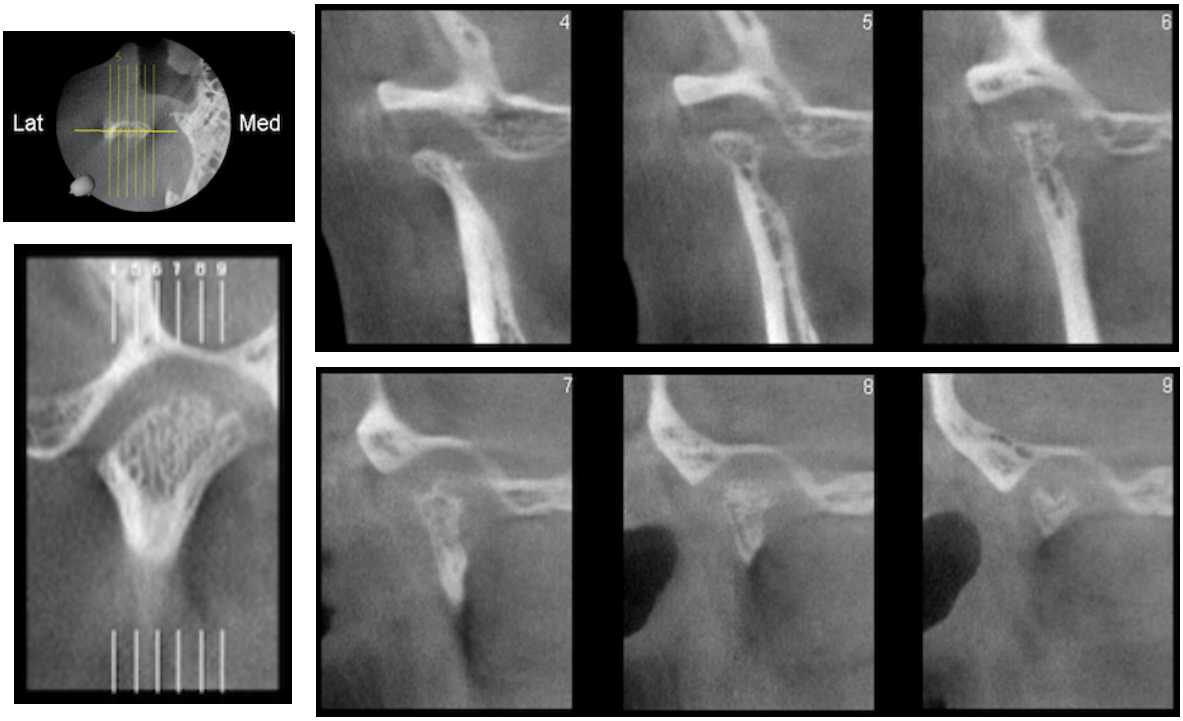
A limitation to our study includes an inability to assess the ICR patient's stage of disease. The saliva samples were collected after condylar resorption had taken place and was visible radiographically. It was difficult to detect whether the subject was experiencing onset of ICR, active stage of ICR, or remission stage. The available method in determining disease stage was the subject's reported history of pain, open lock, locked jaw, and/or joint derangement. We believe that it is unethical to expose patients to CBCT radiation multiple times within a short time span to merely track the changes of condylar resorption for disease stage determination. Therefore, we did not have an accurate method to track ICR disease progression aside from the patients' reported history.

In conclusion, we believe that saliva is a reliable medium to detect cytokine changes in patients with ICR due to its many advantages including easy accessibility, non-invasiveness, safety to patient and researcher, and low cost in diagnostic studies. For future studies, we hope to expand on the pathophysiology of ICR and elucidate the role of each proinflammatory and anti-inflammatory cytokine. Along with salivary diagnostics, we envision the detection of target proteins to evaluate ICR disease risk prior to undergoing various dental procedures, including orthodontic treatment and orthognathic surgery, as well as the determination of condylar resorption risk post-treatment.

FIGURES

Figure 1. CBCT. Patient A

a. Right TMJ lateral closed. Lateral (top left, 4) to medial (bottom right, 9)



b. Right TMJ frontal closed. Anterior (top left, 6) to posterior (bottom right, 11)

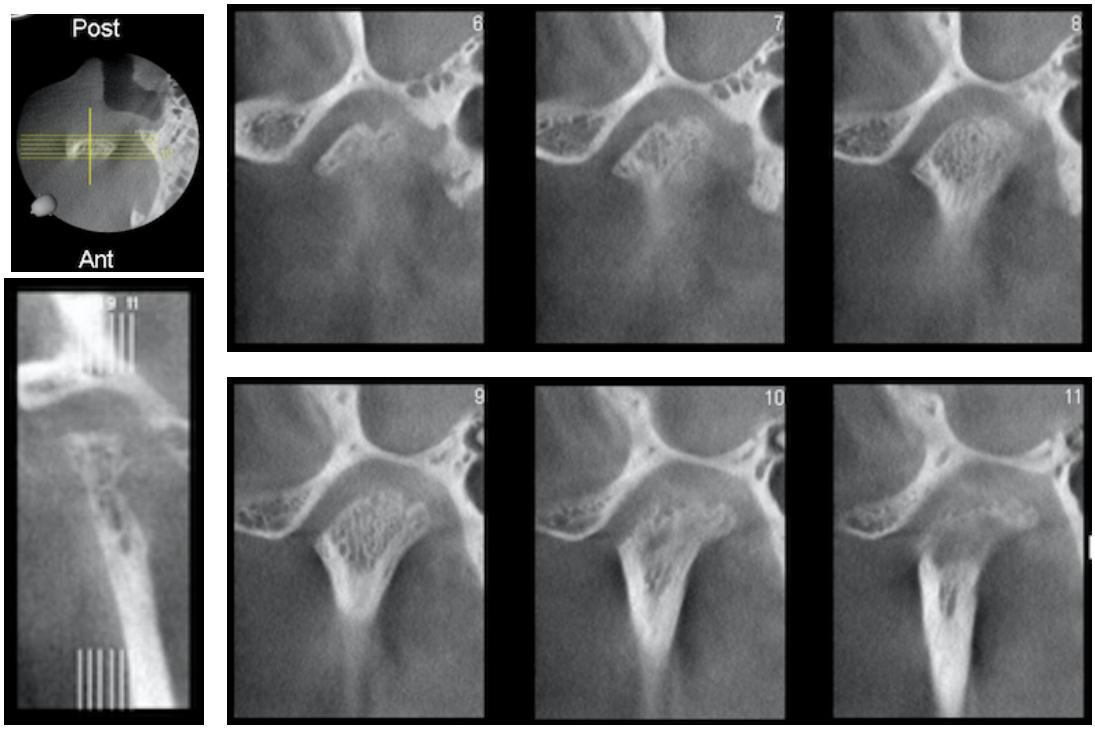
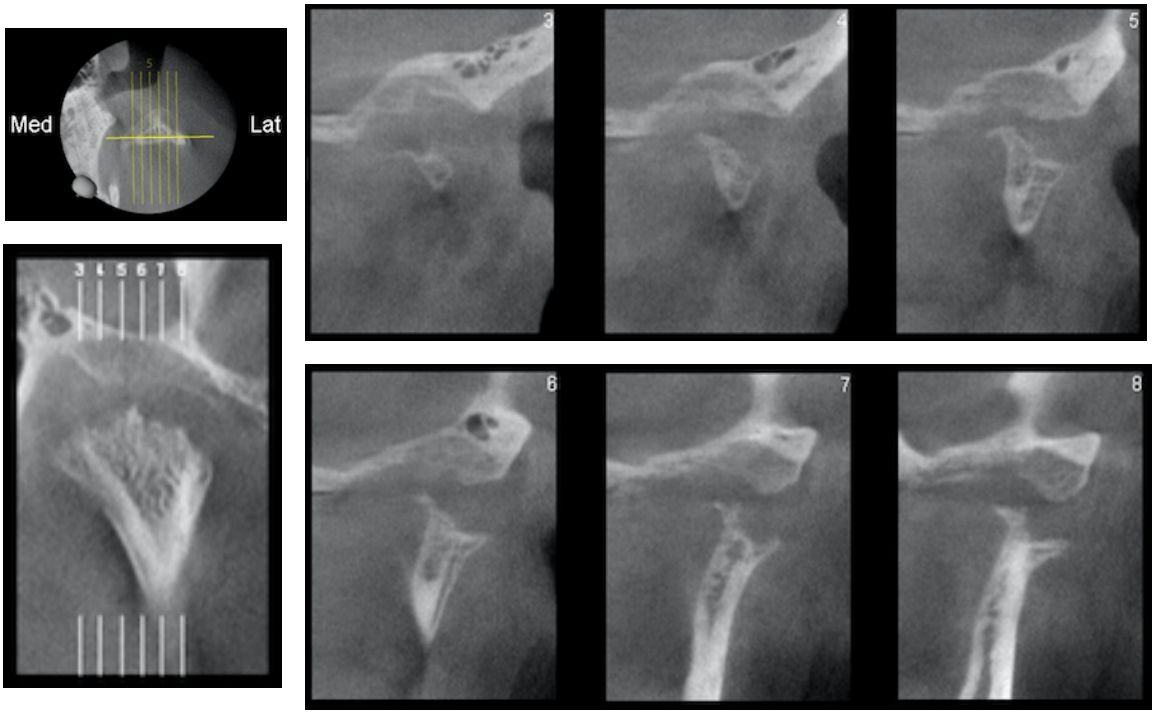


Figure 1 (continued). CBCT. Patient A

c. Left TMJ lateral closed. Medial (top left, 3) to lateral (bottom right, 8)



d. Left TMJ frontal closed. Anterior (top left, 7) to posterior (bottom right, 12)

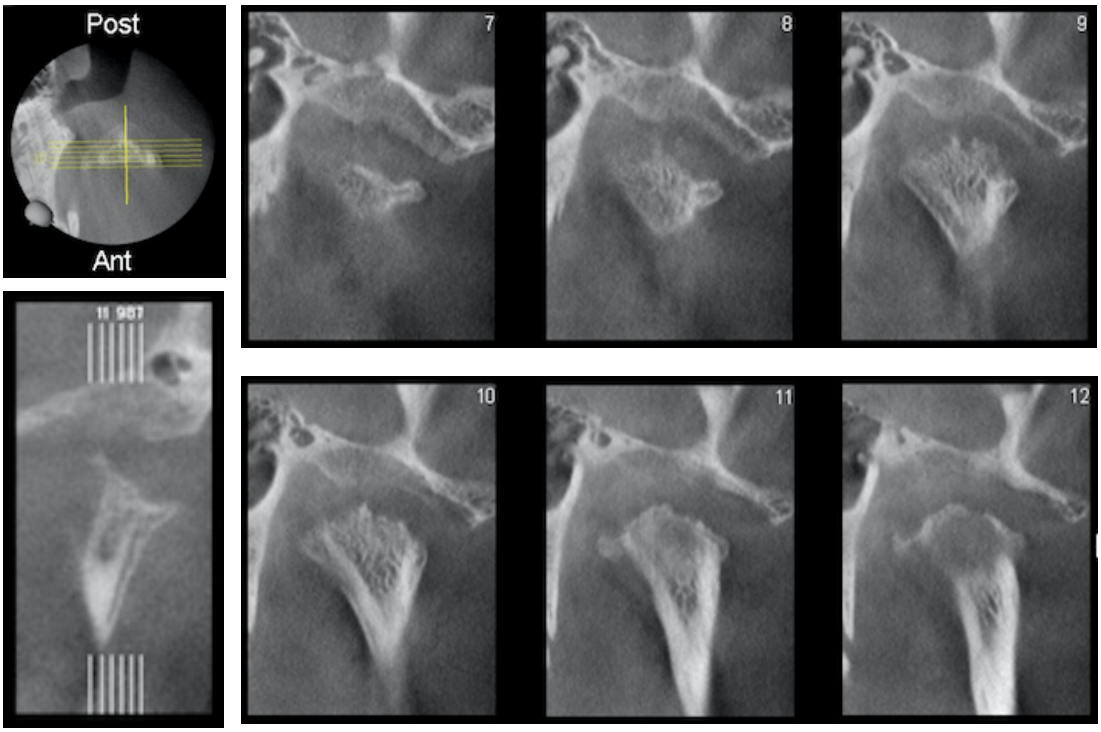
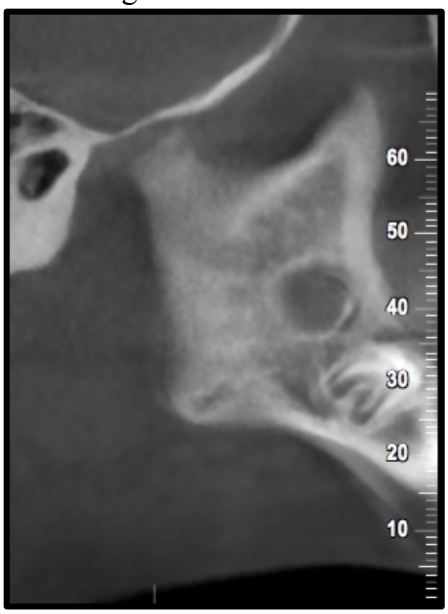
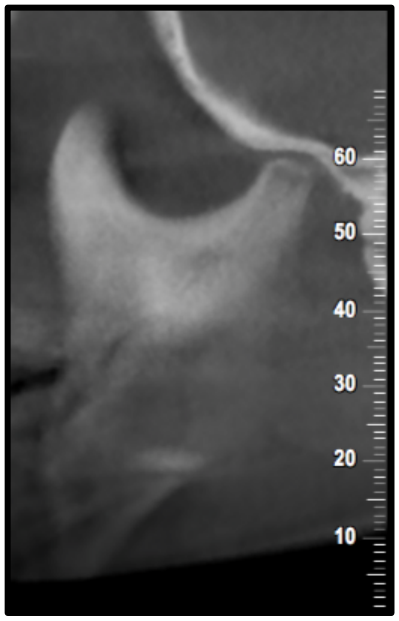


Figure 2. CBCT. Patient B

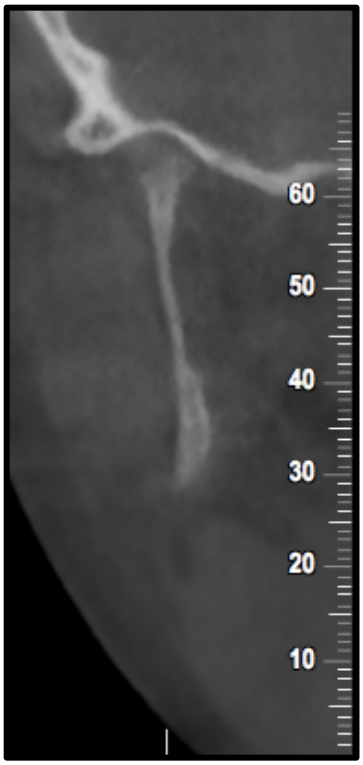
a. CBCT. Right TMJ lateral closed



b. CBCT. Left TMJ lateral closed



c. CBCT. Right TMJ frontal closed



d. CBCT. Left TMJ frontal closed

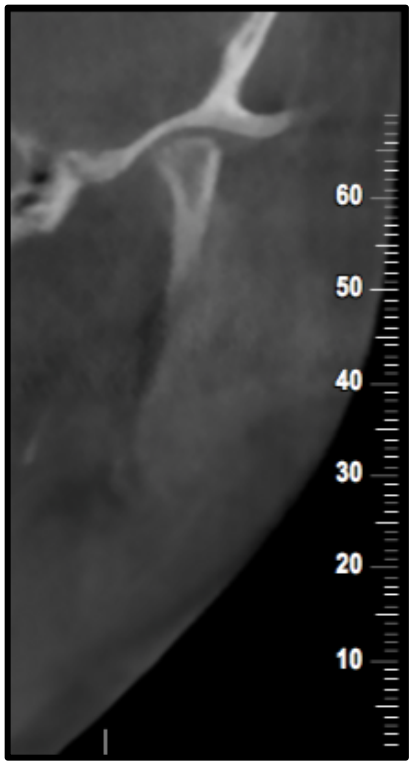
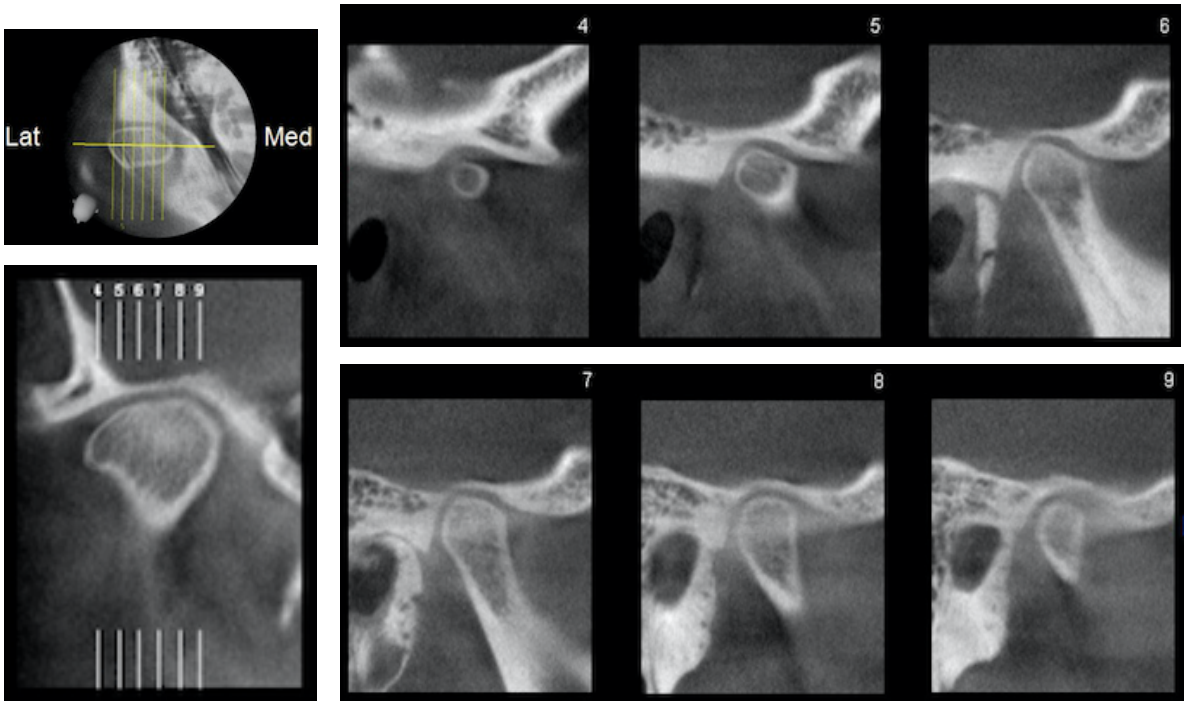


Figure 3. CBCT. Patient C

a. Right TMJ lateral closed. Lateral (top left, 4) to medial (bottom right, 9)



b. Right TMJ frontal closed. Anterior (top left, 9) to posterior (bottom right, 14)

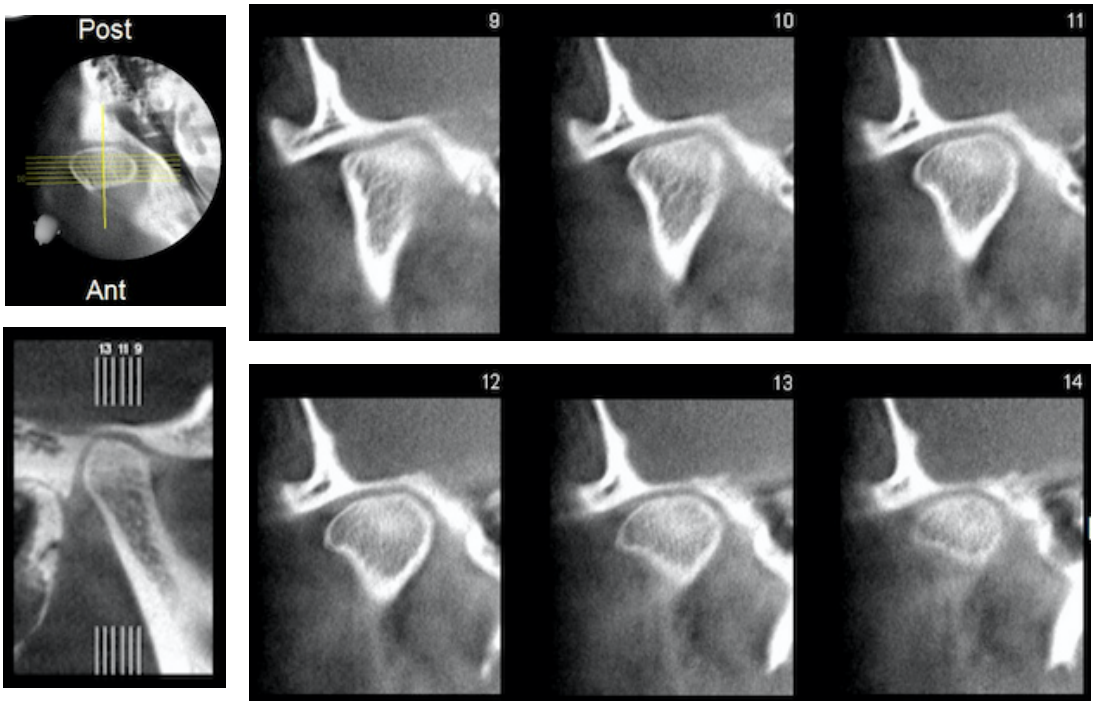
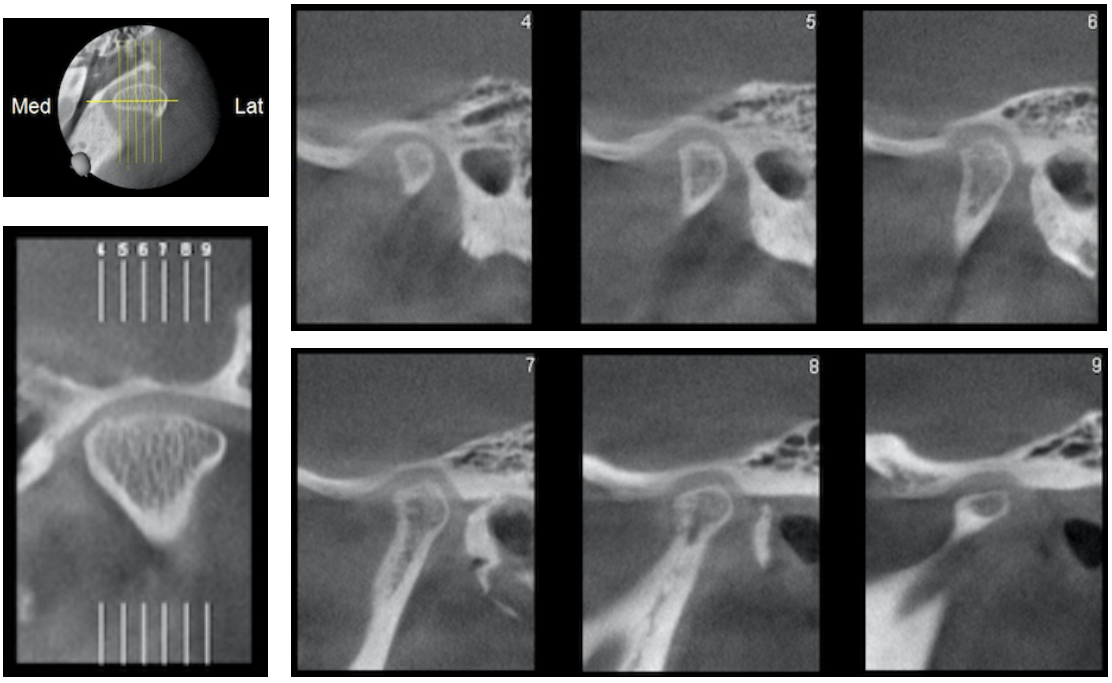


Figure 3 (continued). CBCT. Patient C

c. Left TMJ lateral closed. Medial (top left, 4) to lateral (bottom right, 9)



d. Left TMJ frontal closed. Anterior (top left, 10) to posterior (bottom right, 15)

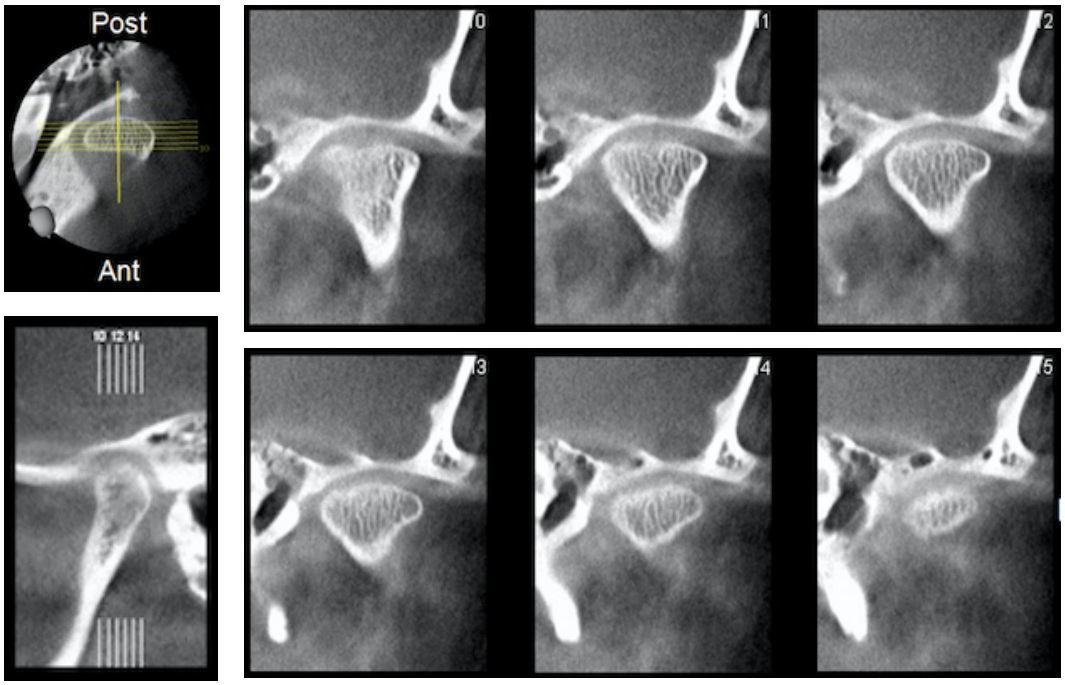
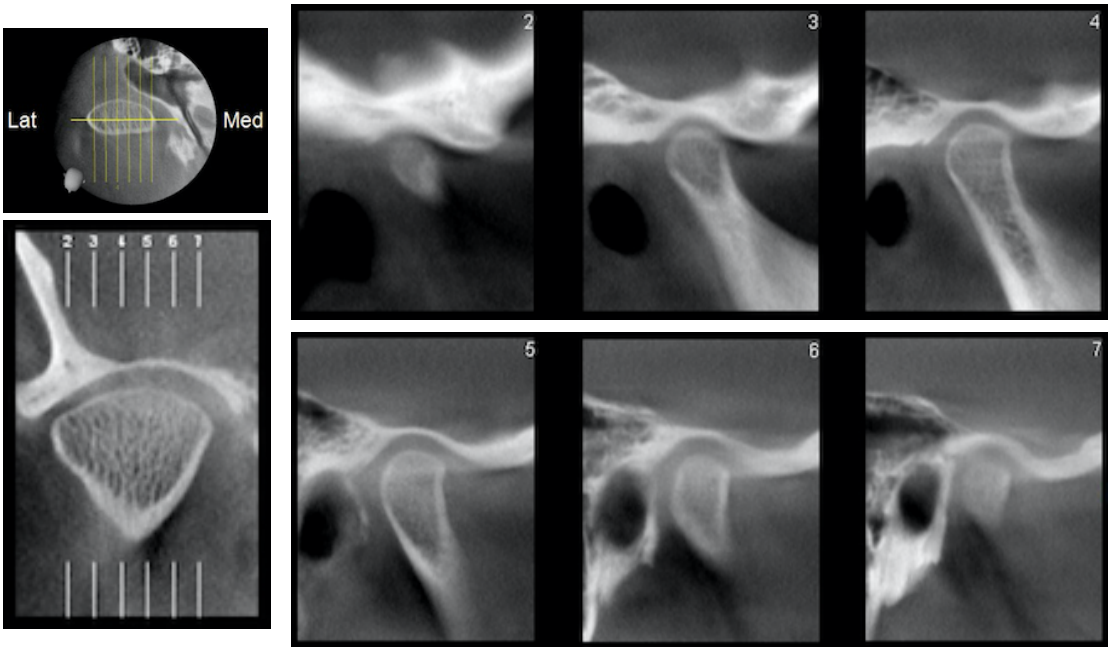


Figure 4. CBCT. Patient D

a. Right TMJ lateral closed. Lateral (top left, 2) to medial (bottom right, 7)



b. Right TMJ frontal closed. Anterior (top left, 5) to posterior (bottom right, 10)

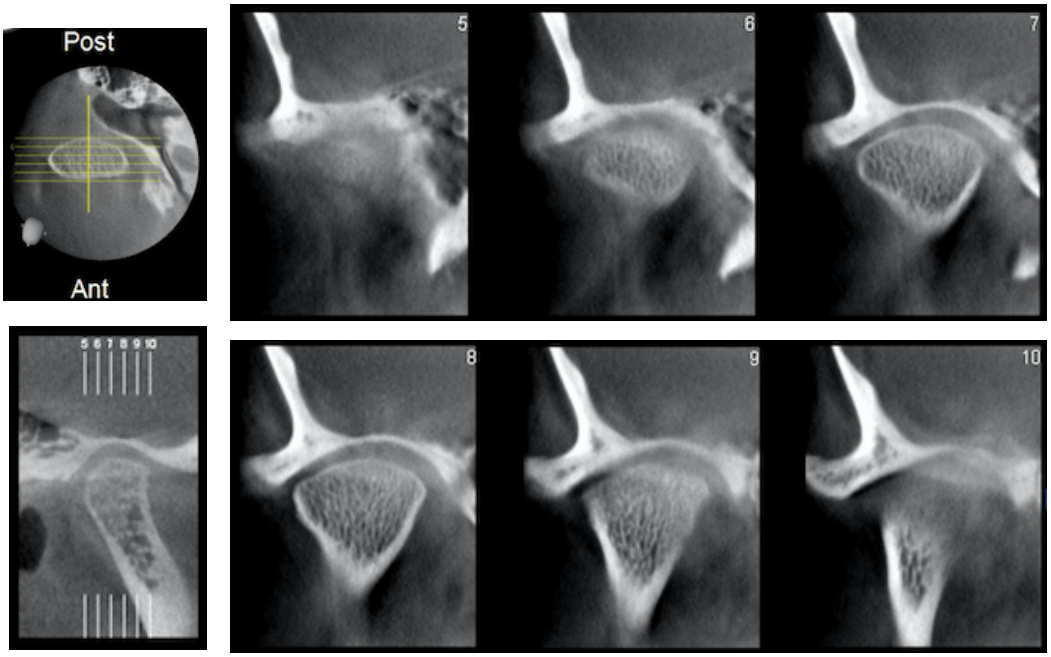
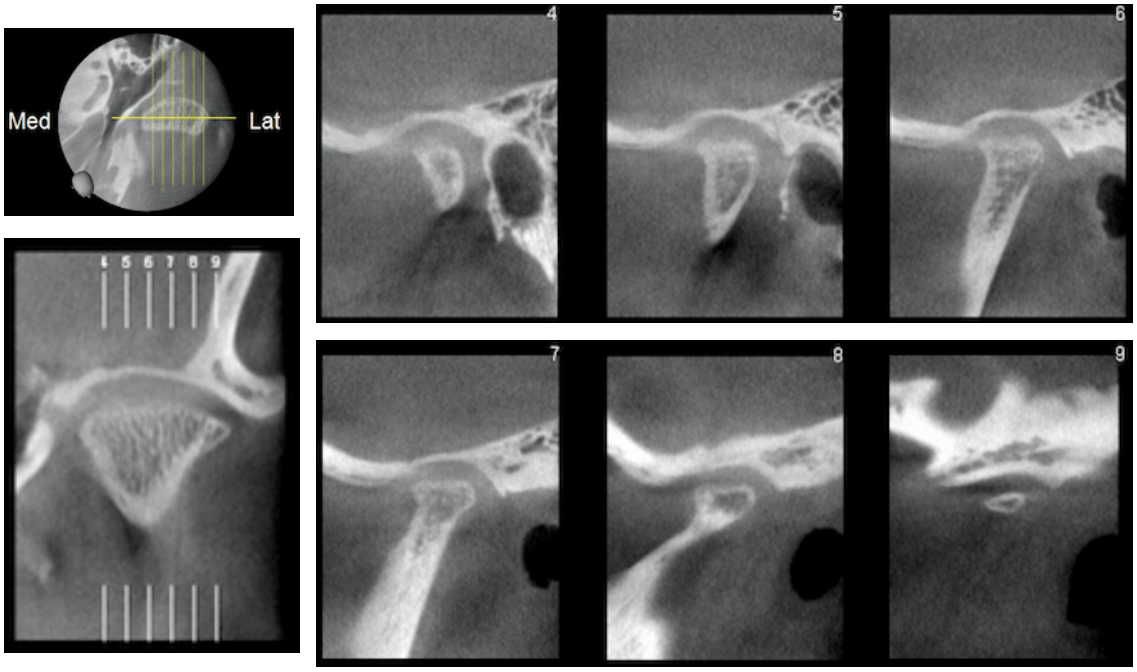


Figure 4 (continued). CBCT. Patient D

c. Left TMJ lateral closed. Medial (top left, 4) to lateral (bottom right, 9)



d. Left TMJ frontal closed. Anterior (top left, 8) to posterior (bottom right, 13)

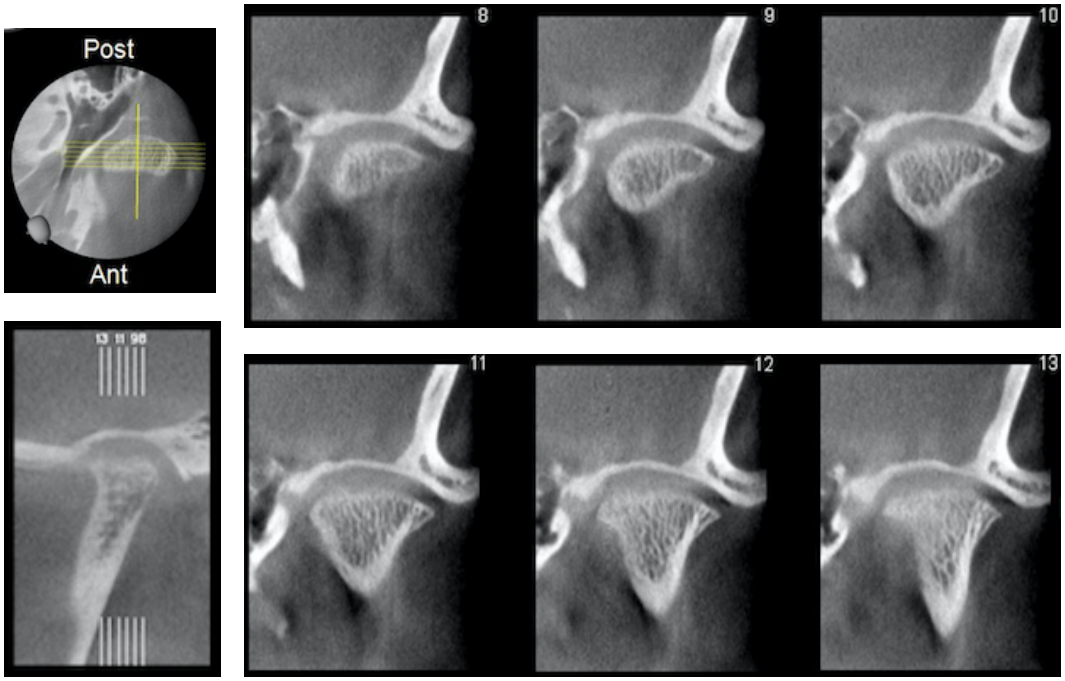
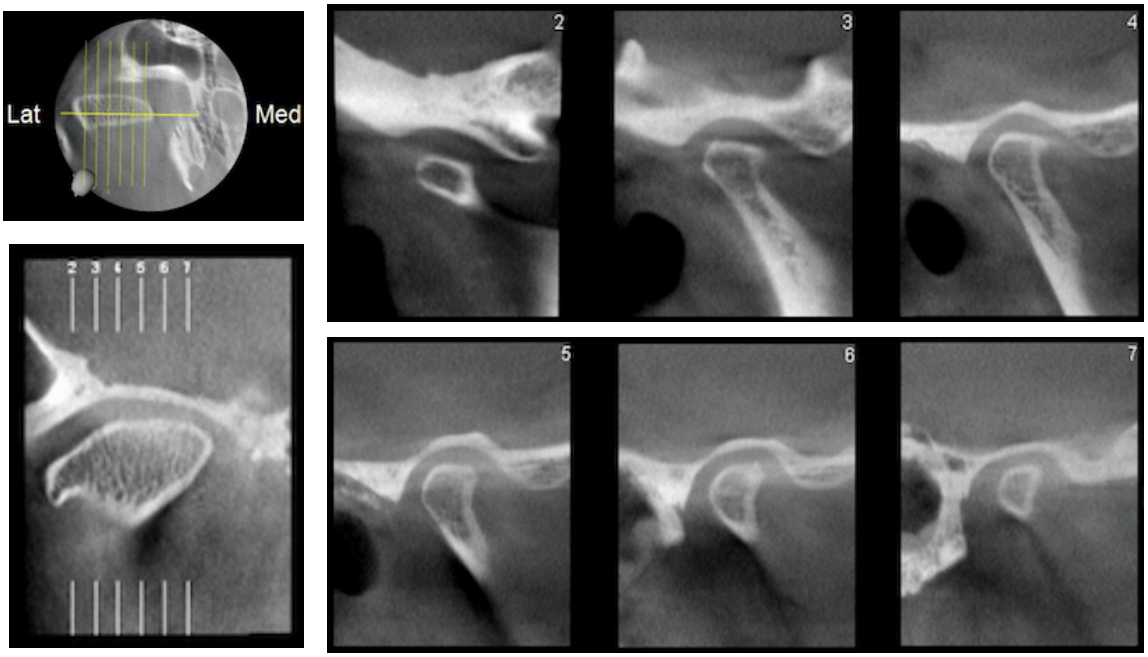


Figure 5. CBCT. Patient E

a. Right TMJ lateral closed. Lateral (top left, 2) to medial (bottom right, 7)



b. Right TMJ frontal closed. Anterior (top left, 14) to posterior (bottom right, 19)

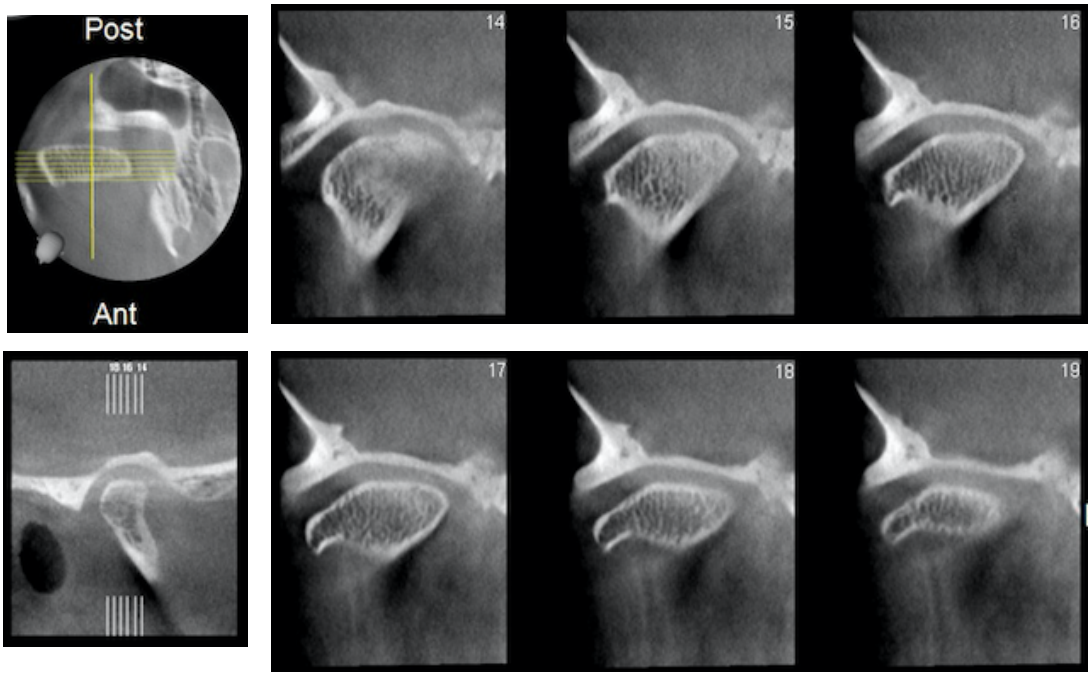
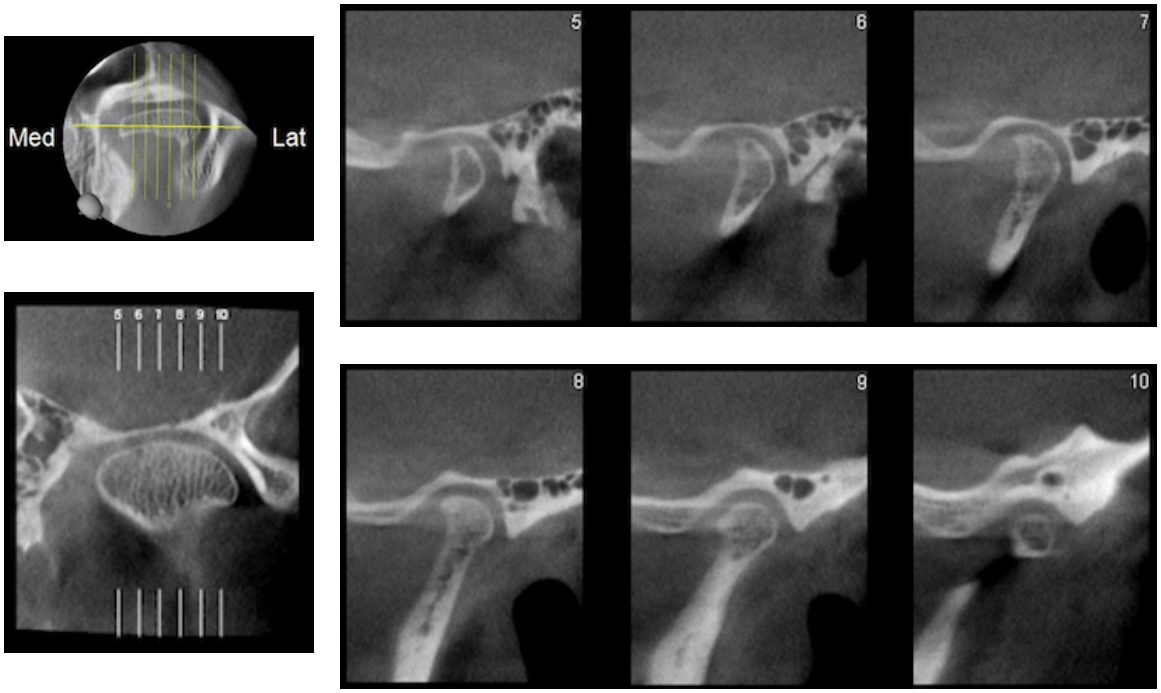


Figure 5 (continued). CBCT. Patient E

c. Left TMJ lateral closed. Medial (top left, 5) to lateral (bottom right, 10)



d. Left TMJ frontal closed. Anterior (top left, 21) to posterior (bottom right, 26)

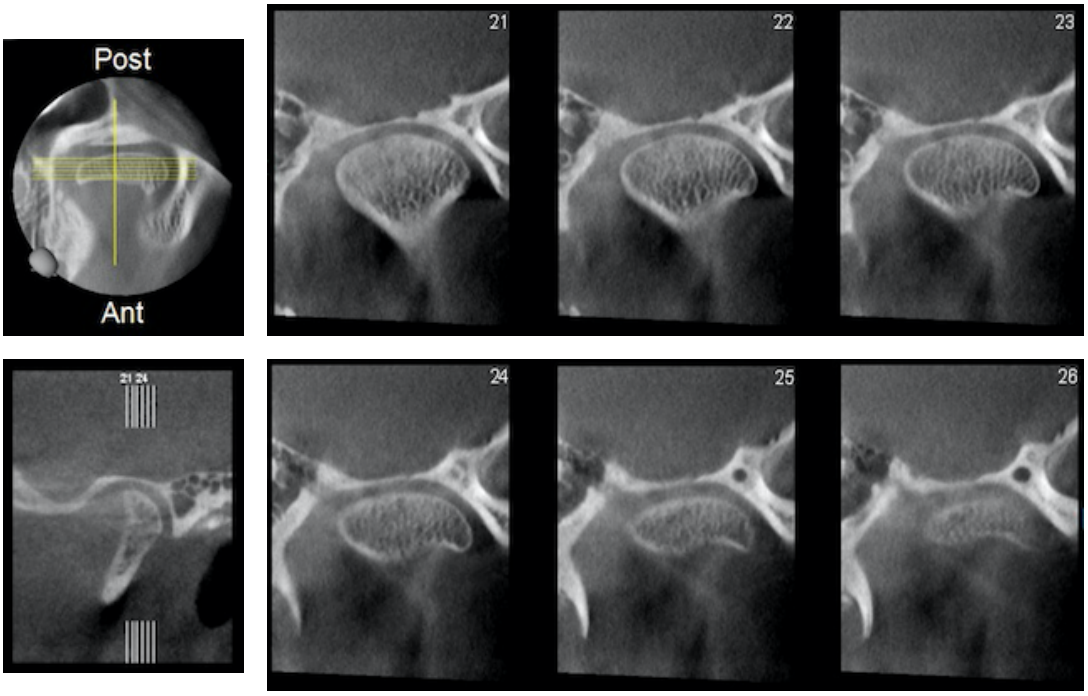
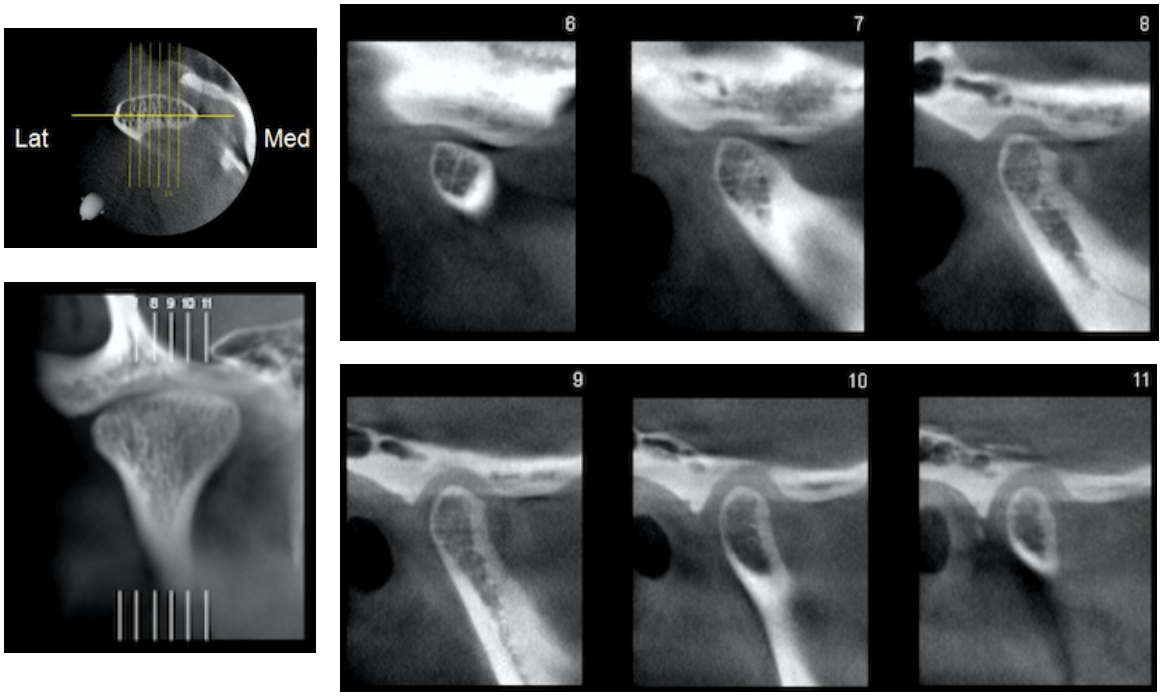


Figure 6. CBCT. Patient F

a. Right TMJ lateral closed. Lateral (top left, 8) to medial (bottom right, 11)



b. Right TMJ frontal closed. Anterior (top left, 10) to posterior (bottom right, 15)

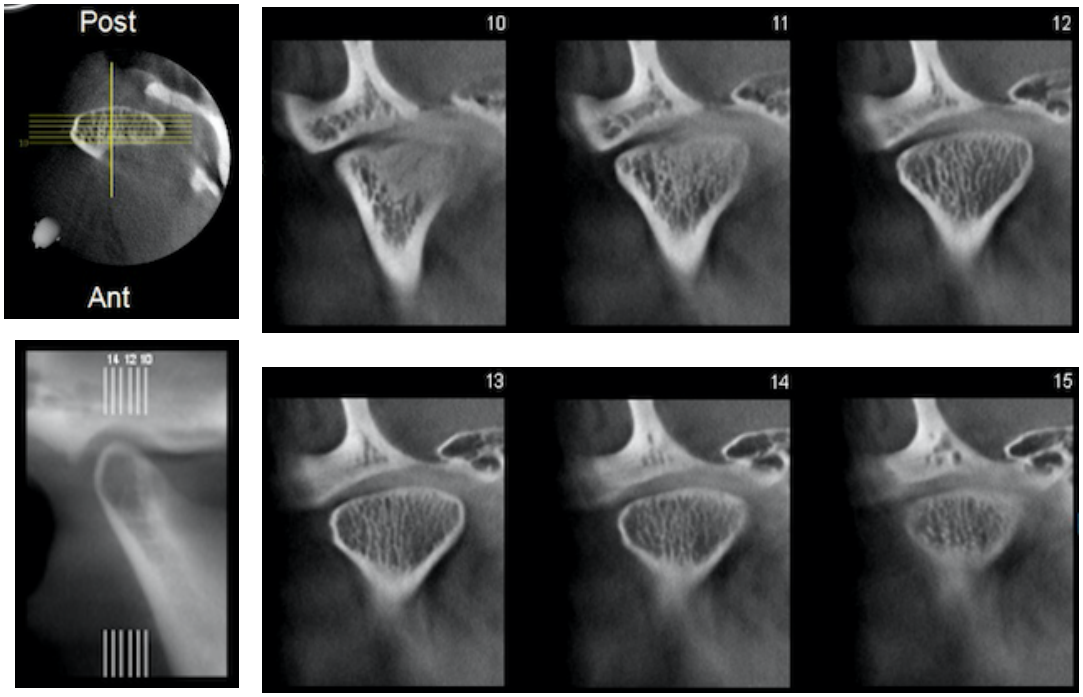
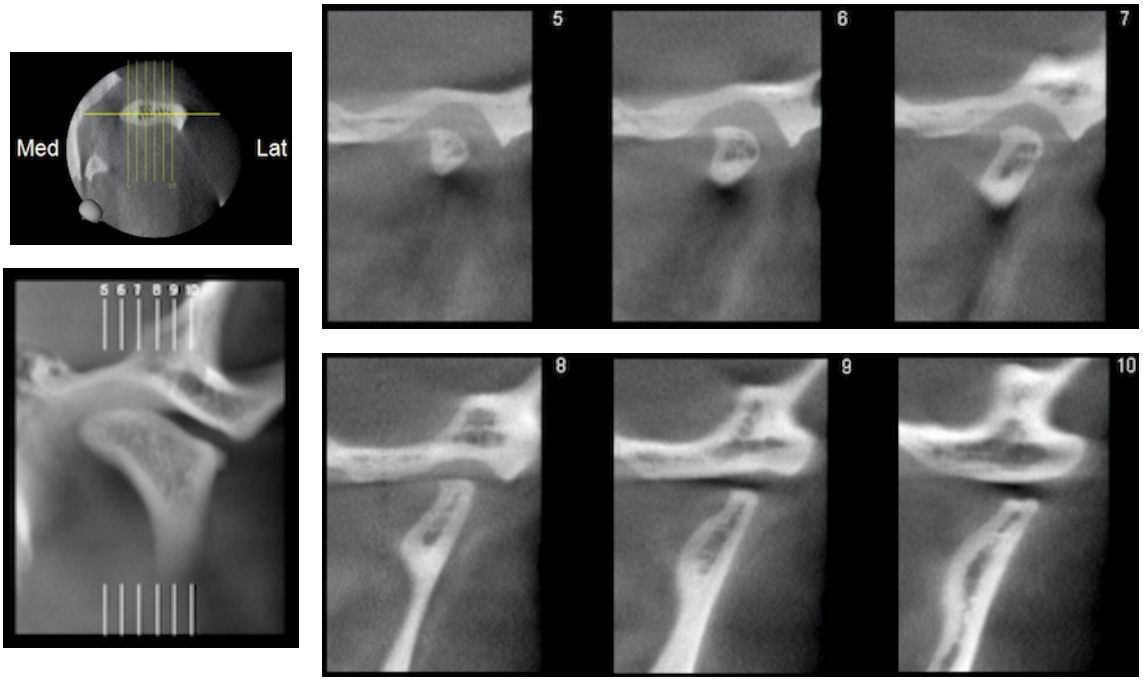


Figure 6 (continued). CBCT. Patient F

c. Left TMJ lateral closed. Medial (top left, 5) to lateral (bottom right, 10)



d. Left TMJ frontal closed. Anterior (top left, 13) to posterior (bottom right, 18)

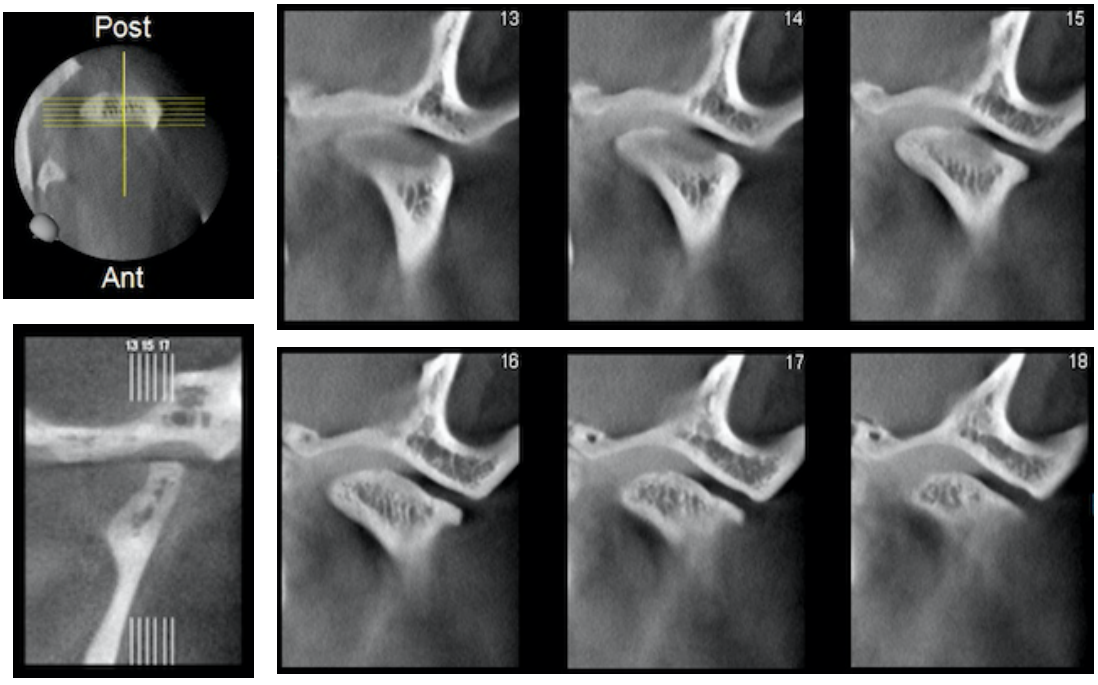
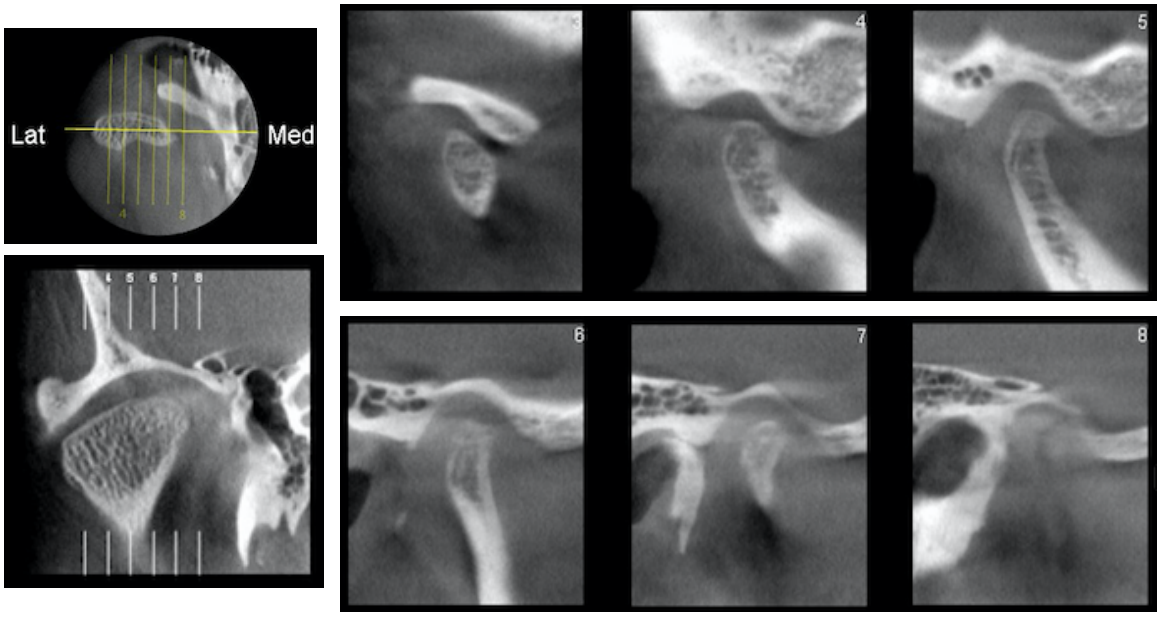


Figure 7. CBCT. Patient G

a. Right TMJ lateral closed. Lateral (top left, 3) to medial (bottom right, 8)



b. Right TMJ frontal closed. Anterior (top left, 18) to posterior (bottom right, 23)

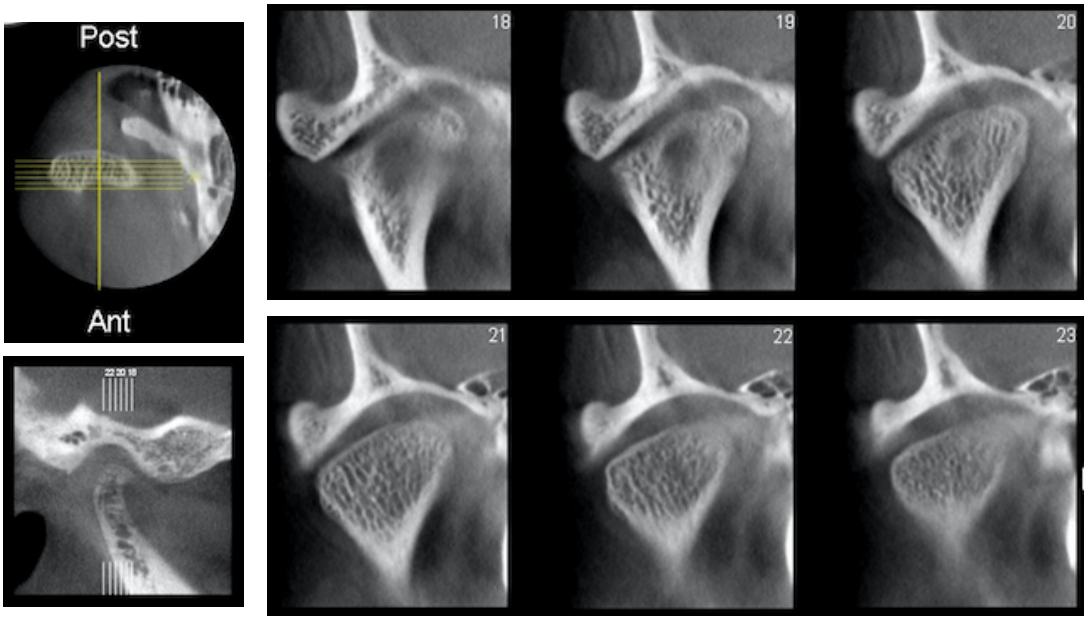
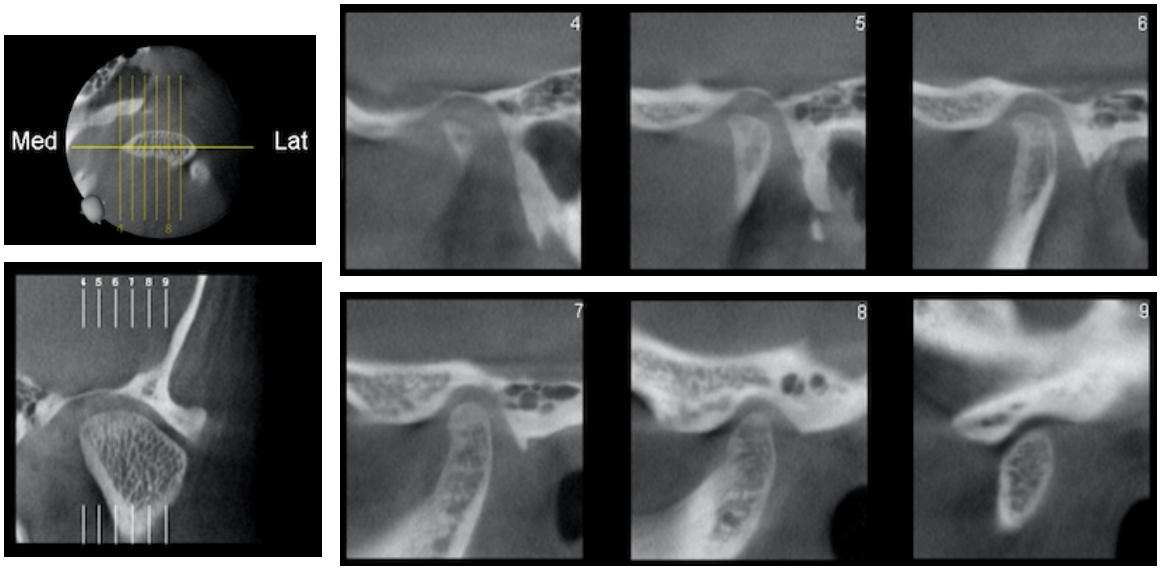


Figure 7 (continued). CBCT. Patient G

c. Left TMJ lateral closed. Medial (top left, 4) to lateral (bottom right, 9)



d. Left TMJ frontal closed. Anterior (top left, 16) to posterior (bottom right, 21)

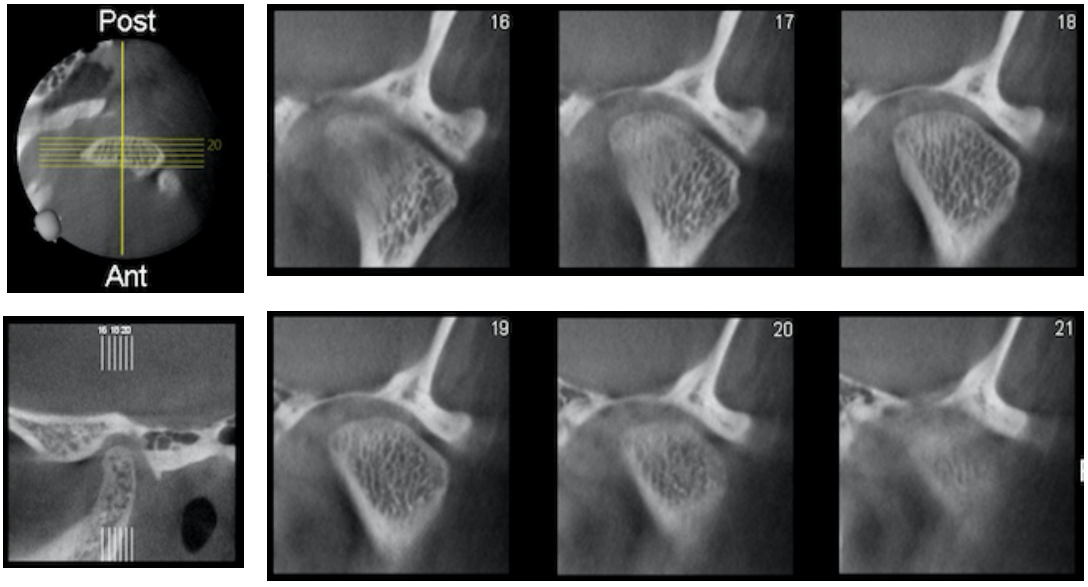
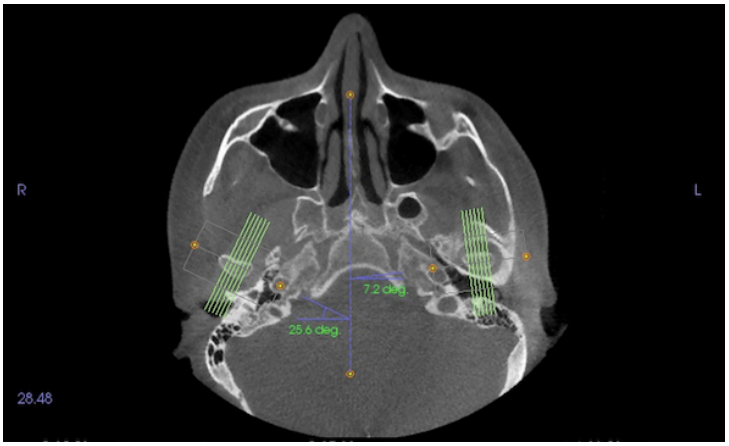
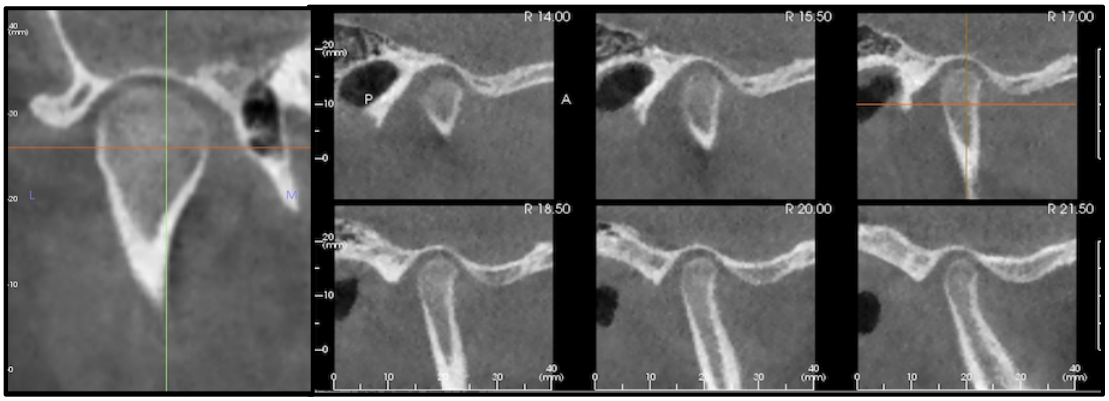


Figure 8. CBCT. Patient H

a. Axial view



b. Right TMJ lateral closed.



c. Left TMJ lateral closed

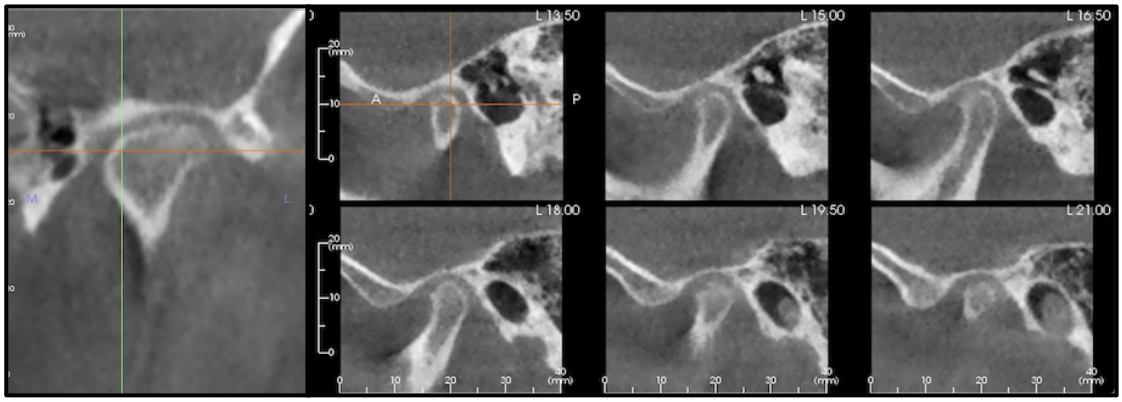
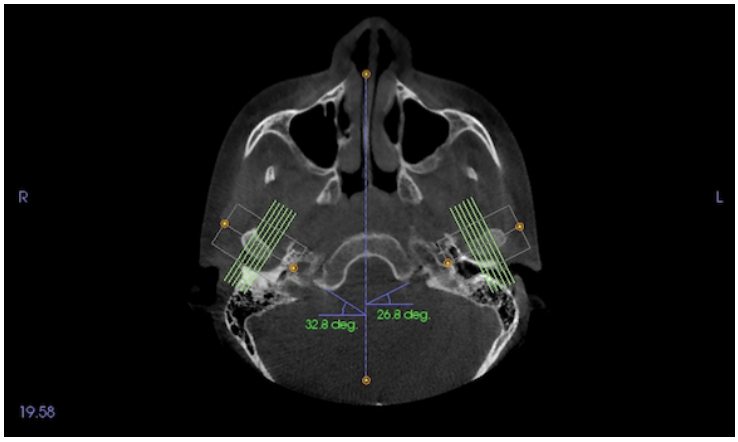
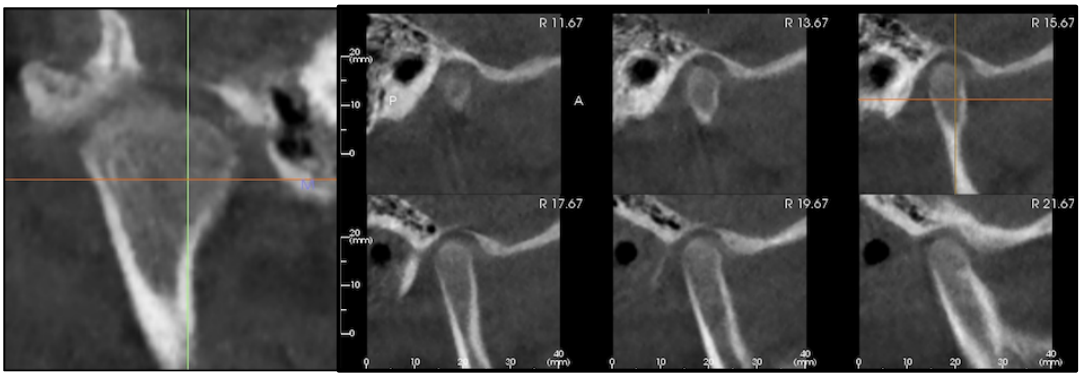


Figure 9. CBCT. Patient I

a. Axial view



b. Right TMJ lateral closed.



c. Left TMJ lateral closed

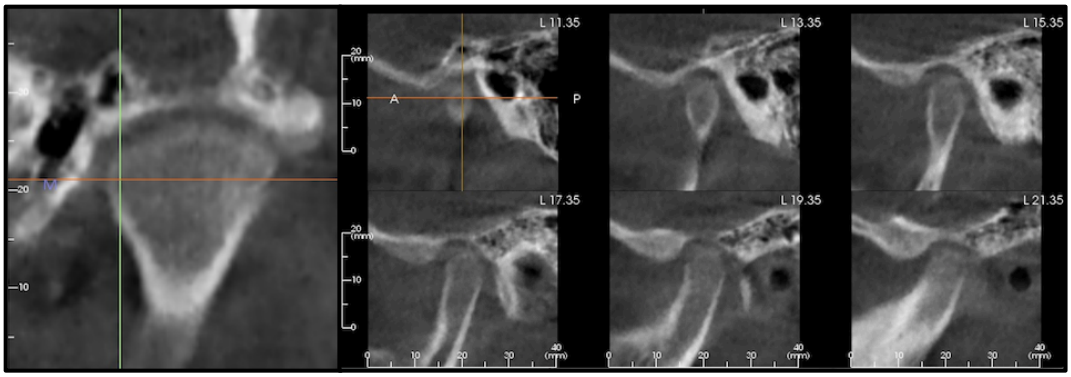
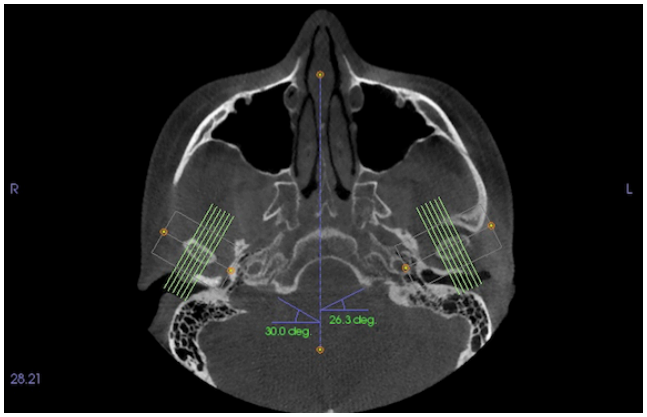
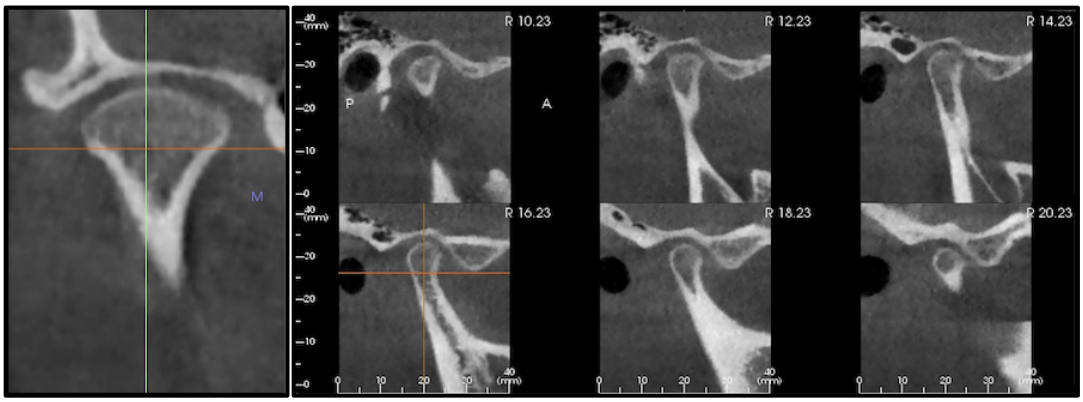


Figure 10. CBCT. Patient J

a. Axial view



b. Right TMJ lateral closed.



c. Left TMJ lateral closed

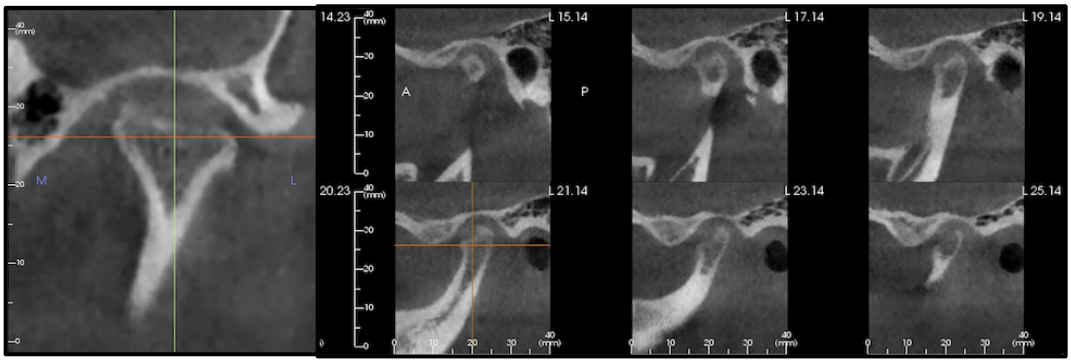
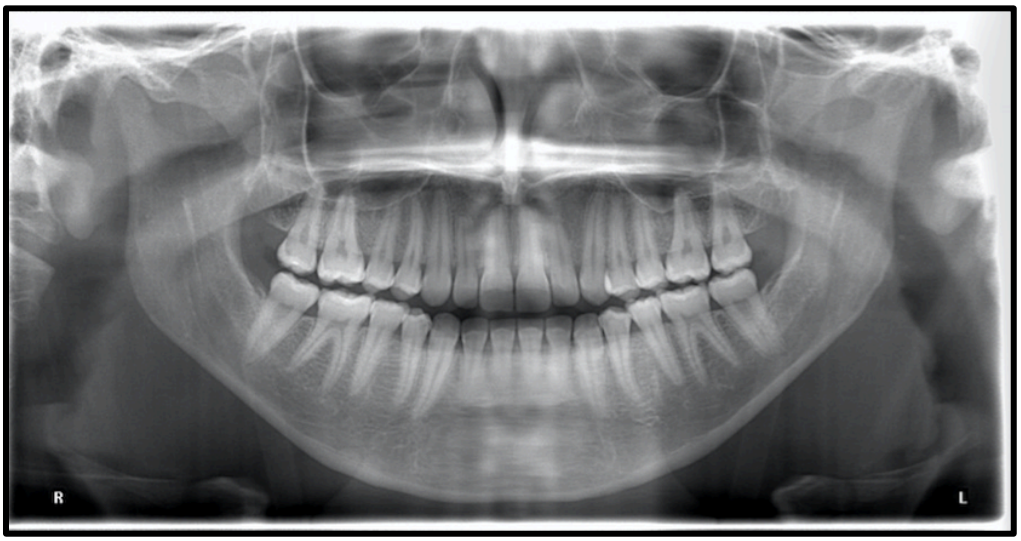
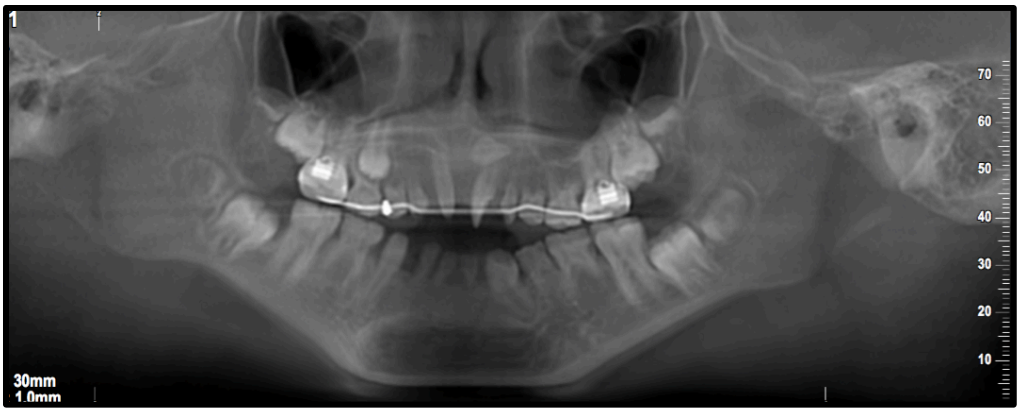


Figure 11. Panoramic radiograph, Patients A, B, E, F, G

a. Patient A



b. Patient B



c. Patient E

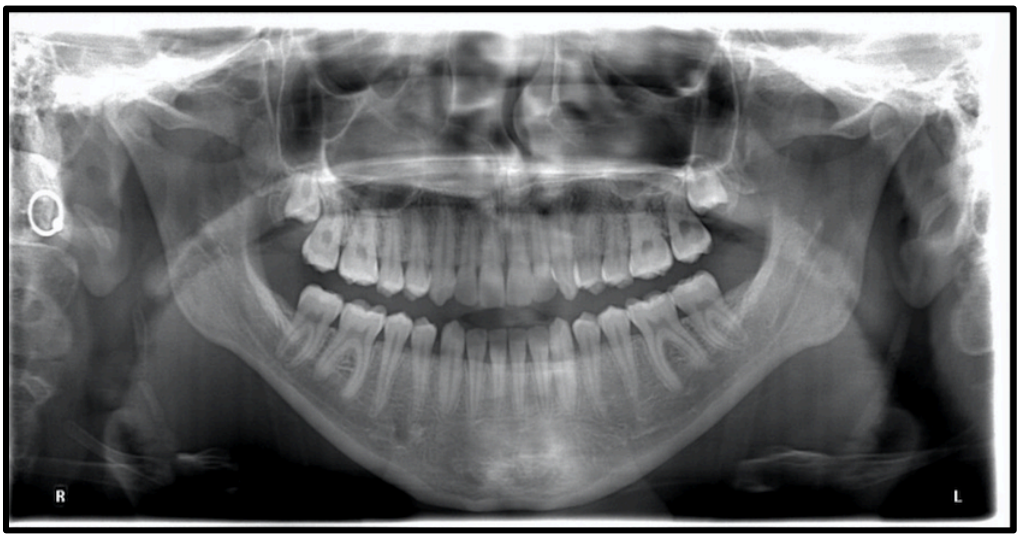
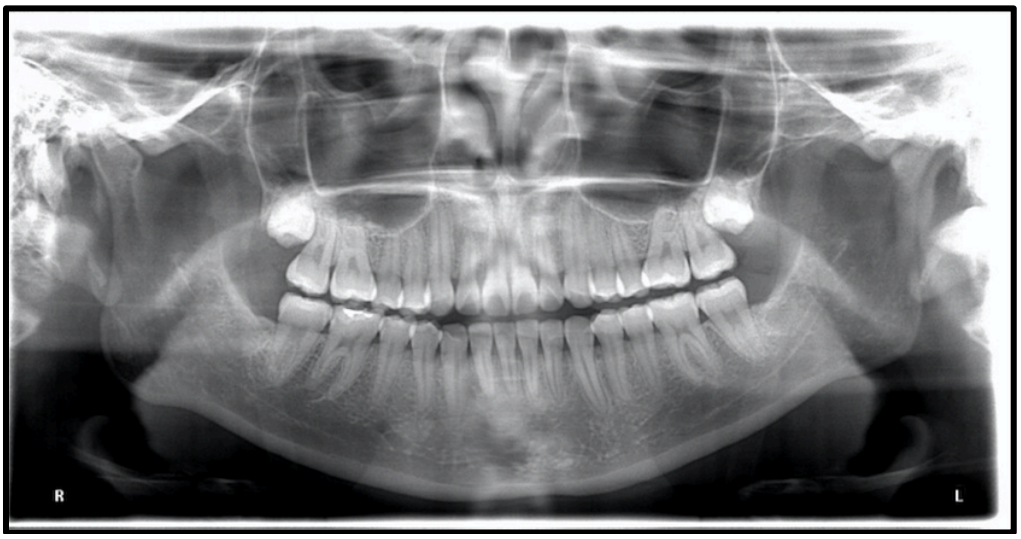


Figure 11 (continued). Panoramic radiograph, Patients A, B, E, F, G

a. Patient F



b. Patient G

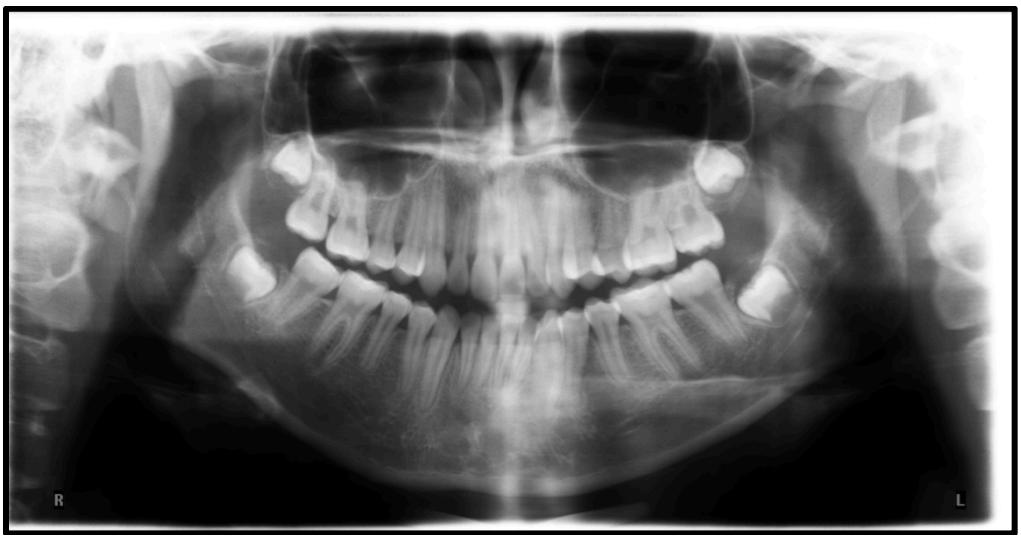
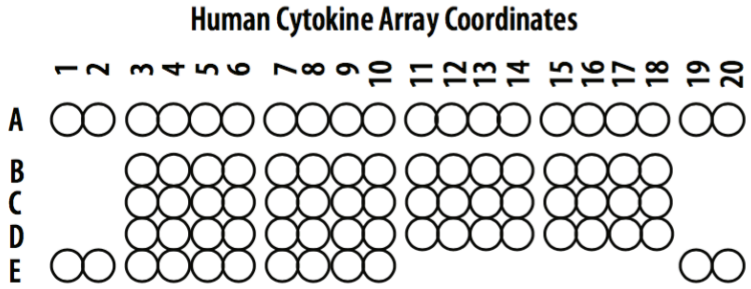


Figure 12. Membrane-based protein array coordinates



Coordinate	Target/Control	Entrez Gene ID	Alternate Nomenclature
A1, A2	Reference Spots	N/A	———
A3, A4	CCL1/I-309	6346	P500, SCYA1, SCYA2, TCA-3
A5, A6	CCL2/MCP-1	6347	MCAF
A7, A8	MIP-1α/MIP-1β	6348/6351	CCL3/CCL4
A9, A10	CCL5/RANTES	6352	———
A11, A12	CD40 Ligand/TNFSF5	959	CD154, CD40LG, gp39, TRAP
A13, A14	Complement Component C5/C5a	727	C5/C5a
A15, A16	CXCL1/GROα	2919	CINC-1, KC
A17, A18	CXCL10/IP-10	3627	CRG-2
A19, A20	Reference Spots	N/A	———
B3, B4	CXCL11/I-TAC	6373	β-R1, H174
B5, B6	CXCL12/SDF-1	6387	PBSF
B7, B8	G-CSF	1440	CSFβ, CSF-3
B9, B10	GM-CSF	1437	CSFα, CSF-2
B11, B12	ICAM-1/CD54	3383	———
B13, B14	IFN-γ	3458	Type II IFN
B15, B16	IL-1α/IL-1F1	3552	———
B17, B18	IL-1β/IL-1F2	3553	———
C3, C4	IL-1ra/IL-1F3	3557	———
C5, C6	IL-2	3558	TCGF
C7, C8	IL-4	3565	BCDF, BSF1
C9, C10	IL-5	3567	———
C11, C12	IL-6	3569	BSF-2
C13, C14	IL-8	3576	CXCL8, GCP1, NAP1
C15, C16	IL-10	3586	CSIF
C17, C18	IL-12 p70	3592/3593	CLMF p35

Figure 12 (continued). Membrane-based protein array coordinates

Coordinate	Target/Control	Entrez Gene ID	Alternate Nomenclature
D3, D4	IL-13	3596	———
D5, D6	IL-16	3603	LCF
D7, D8	IL-17A	3605	CTLA-8
D9, D10	IL-17E	64806	IL-25
D11, D12	IL-18/IL-1F4	3606	IGIF
D13, D14	IL-21	59067	———
D15, D16	IL-27	246778	IL-27 A
D17, D18	IL-32α	9235	
E1, E2	Reference Spots	N/A	———
E3, E4	MIF	4282	GIF, DER6
E5, E6	Serpin E1/PAI-1	5054	Nexin, PLANH1
E7, E8	TNF-α	7124	TNFSF1A
E9, E10	TREM-1	54210	CD354
E19, E20	Negative Control	N/A	———

Figure 13. Membrane-based protein array. Patient A – J = ICR

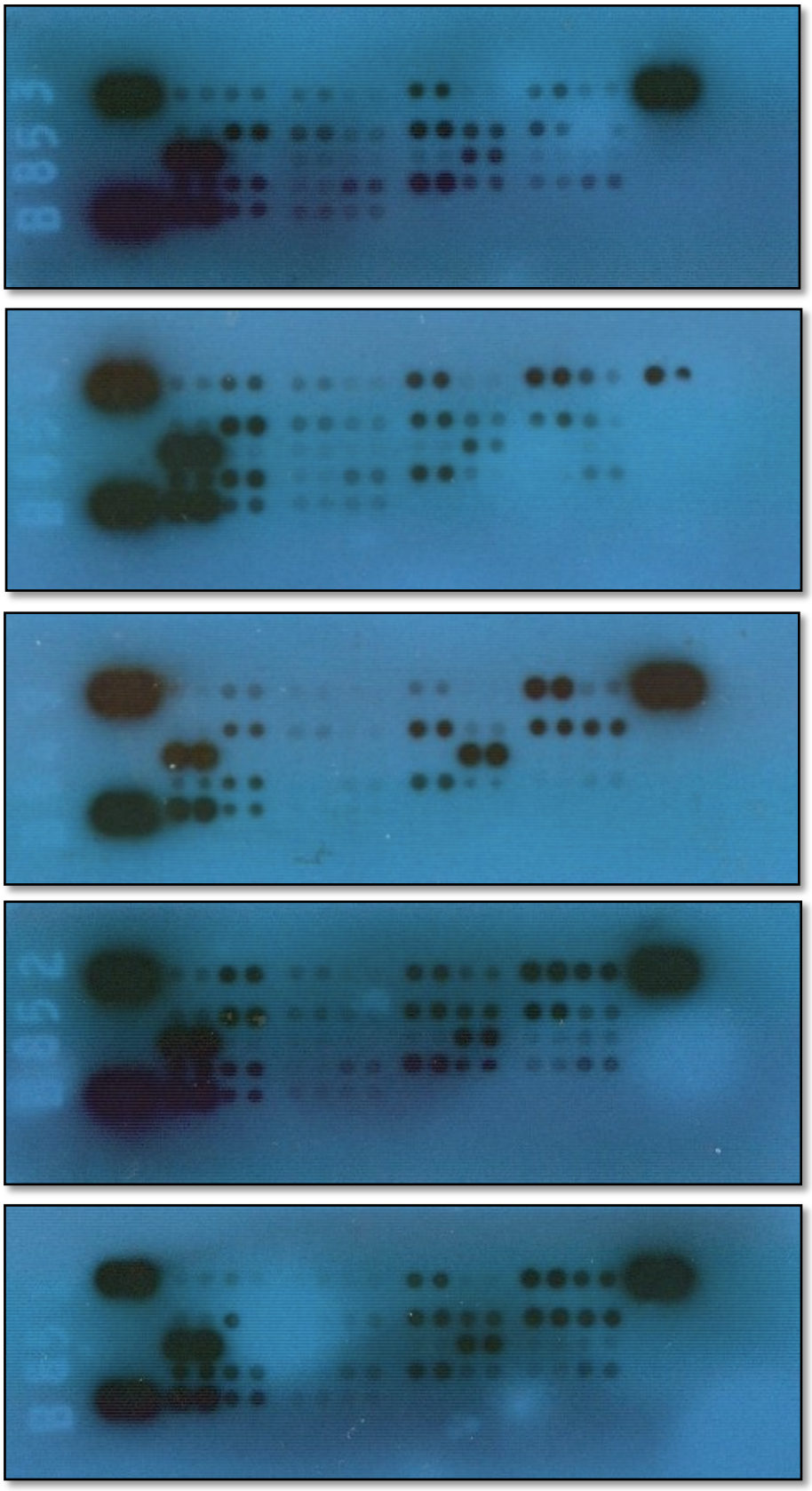


Figure 13. Membrane-based protein array. Patient A – J = ICR

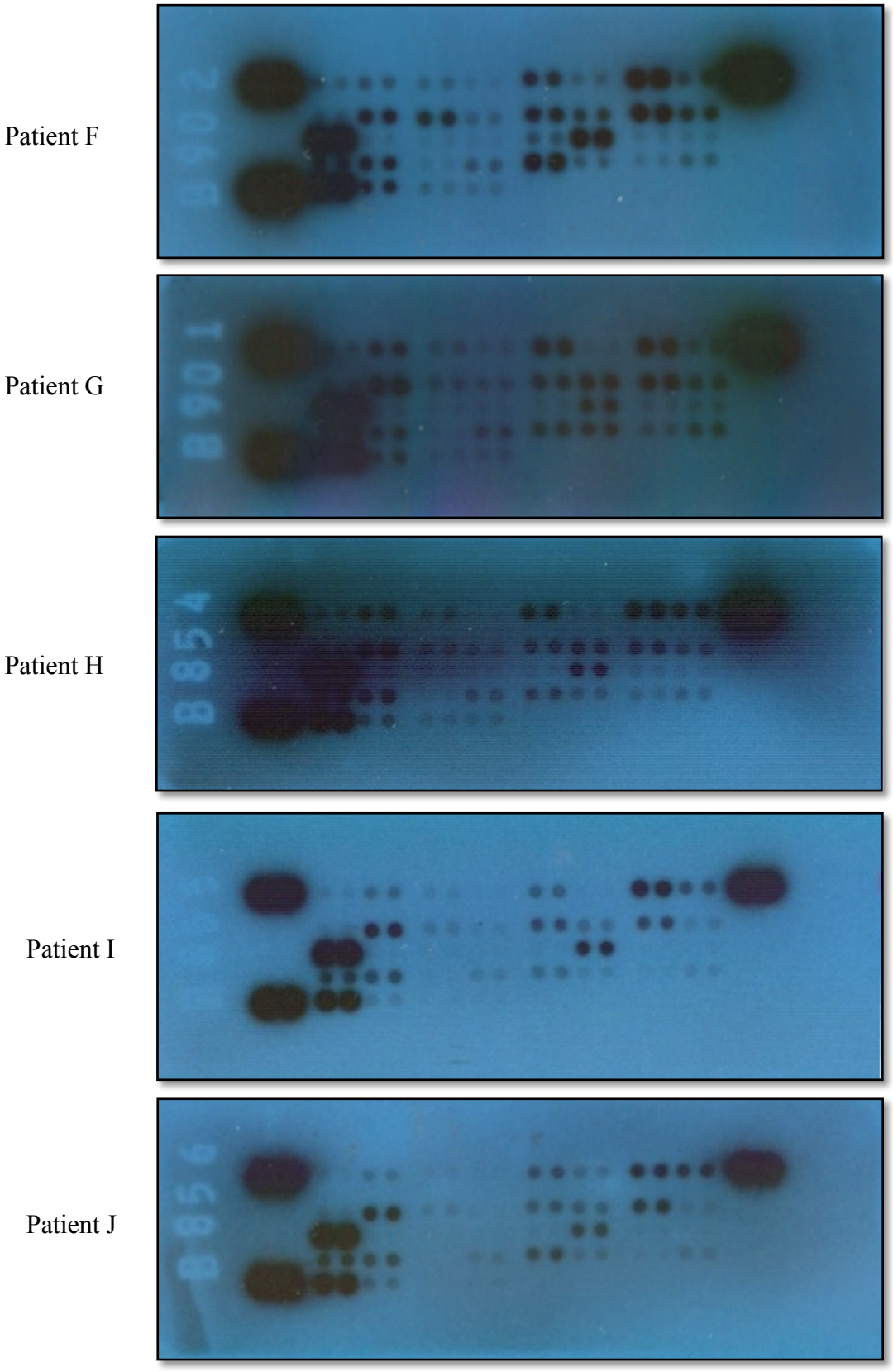


Figure 14. Membrane-based protein array. Control A – J = Control

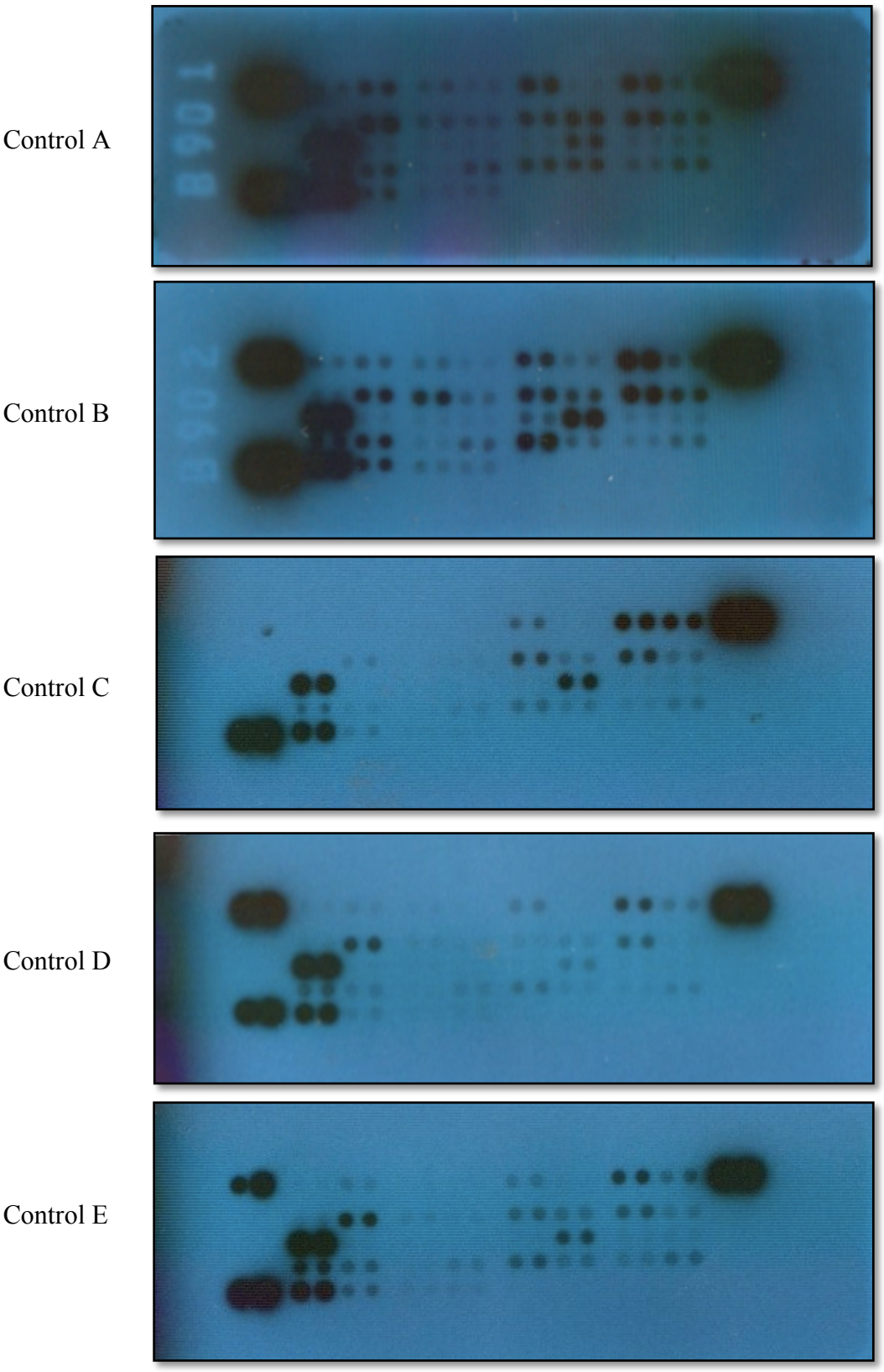


Figure 14. Membrane-based protein array. Control A – J = Control

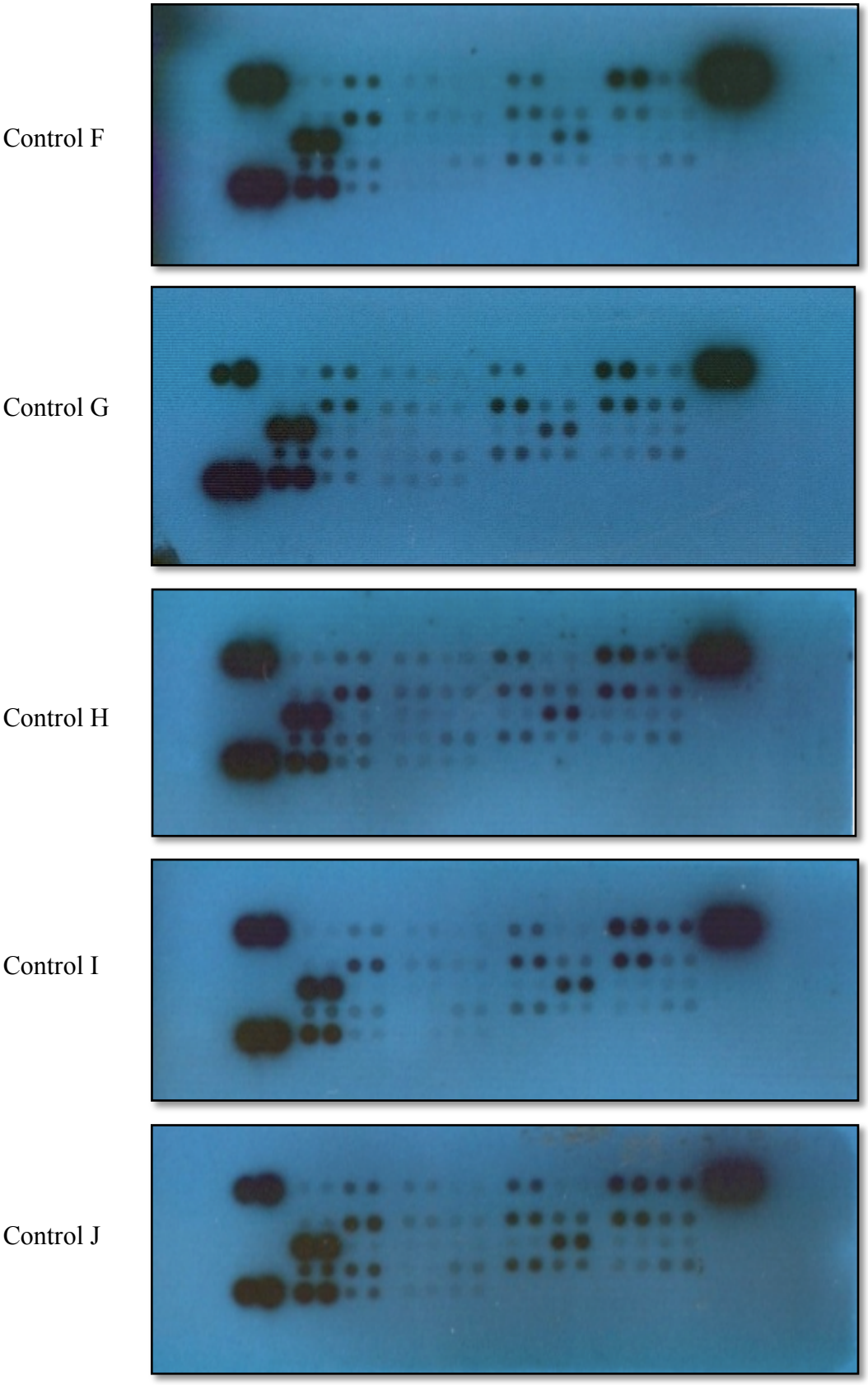
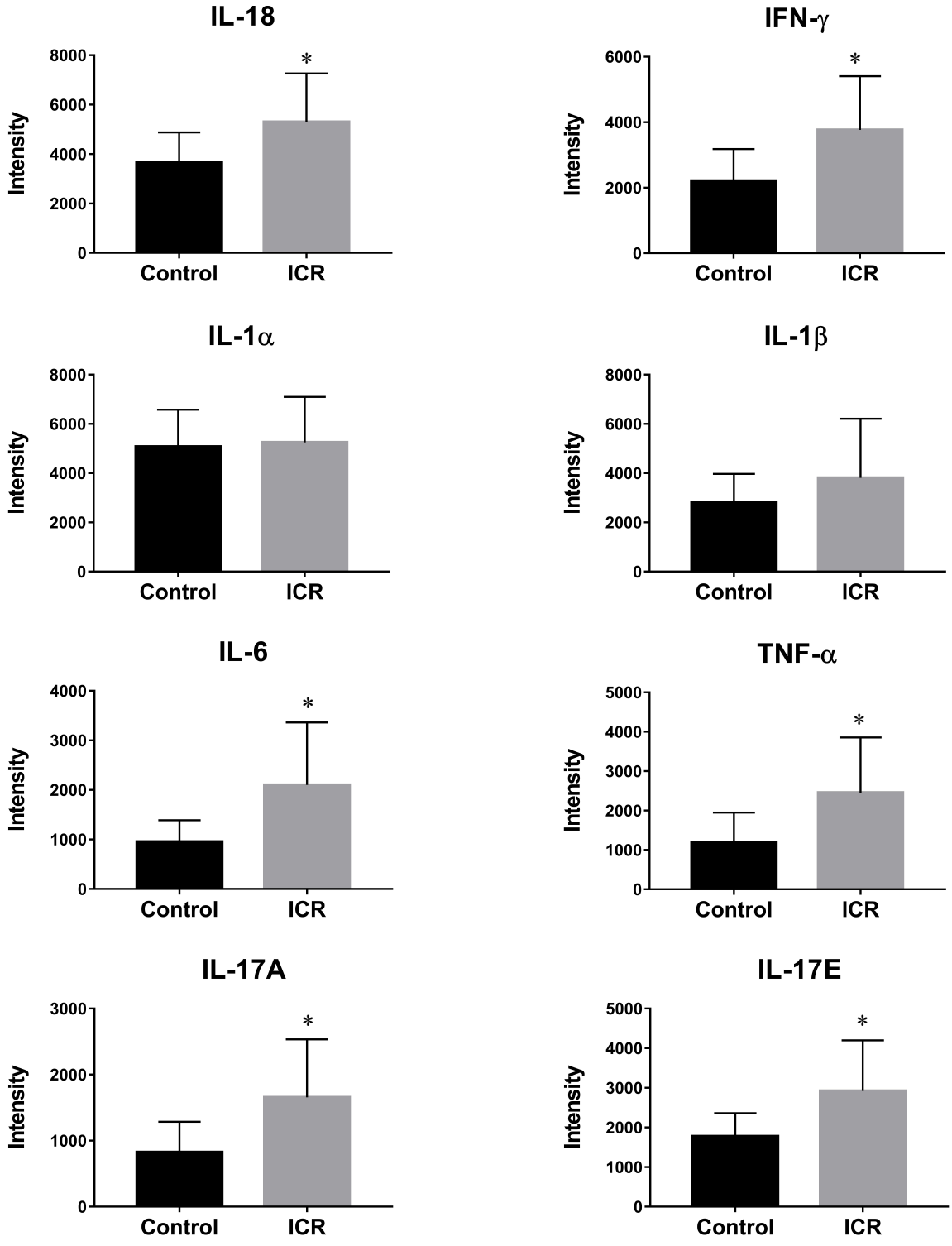
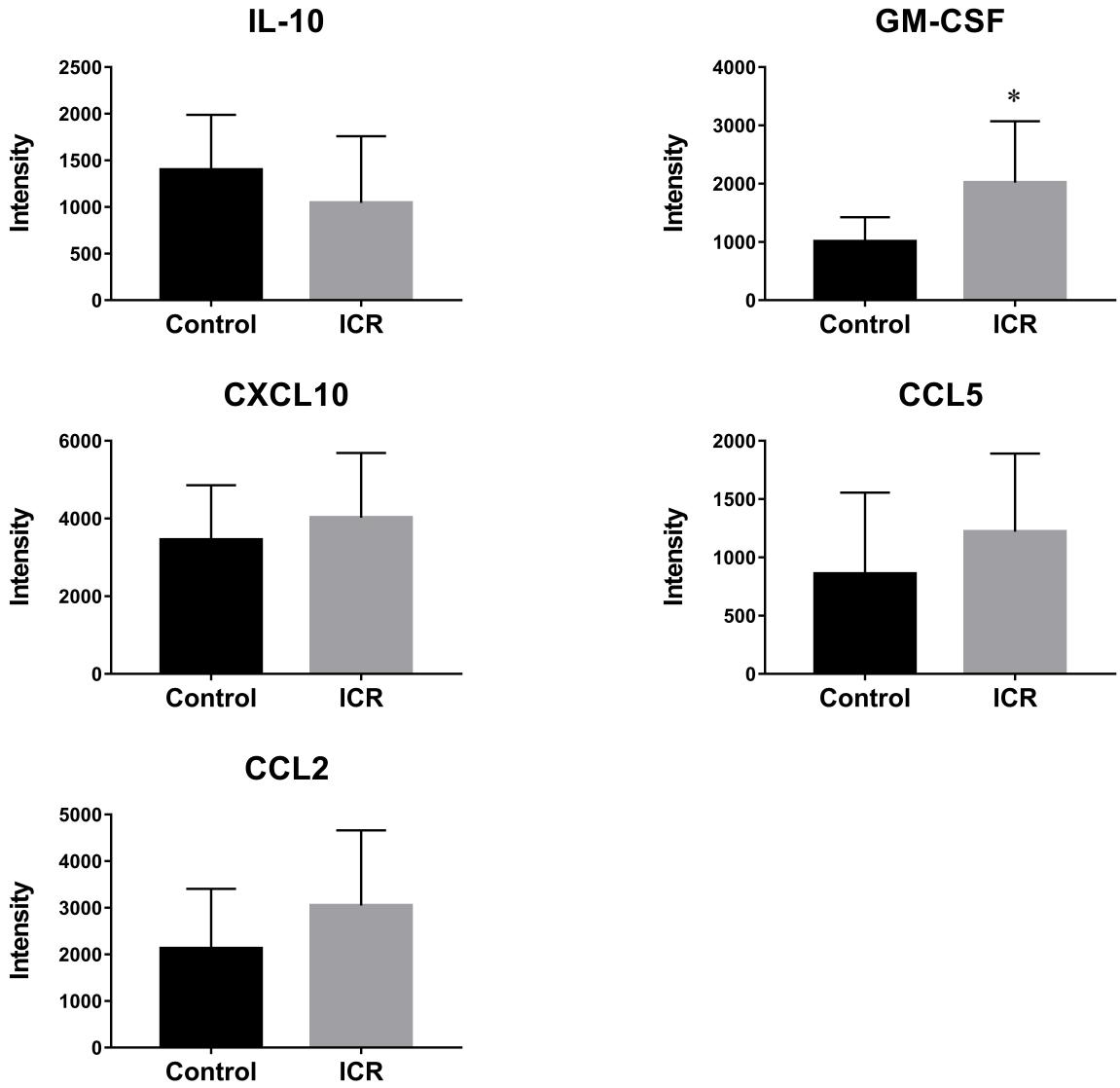


Figure 15. Cytokine intensity in control versus ICR (Bar Graph)



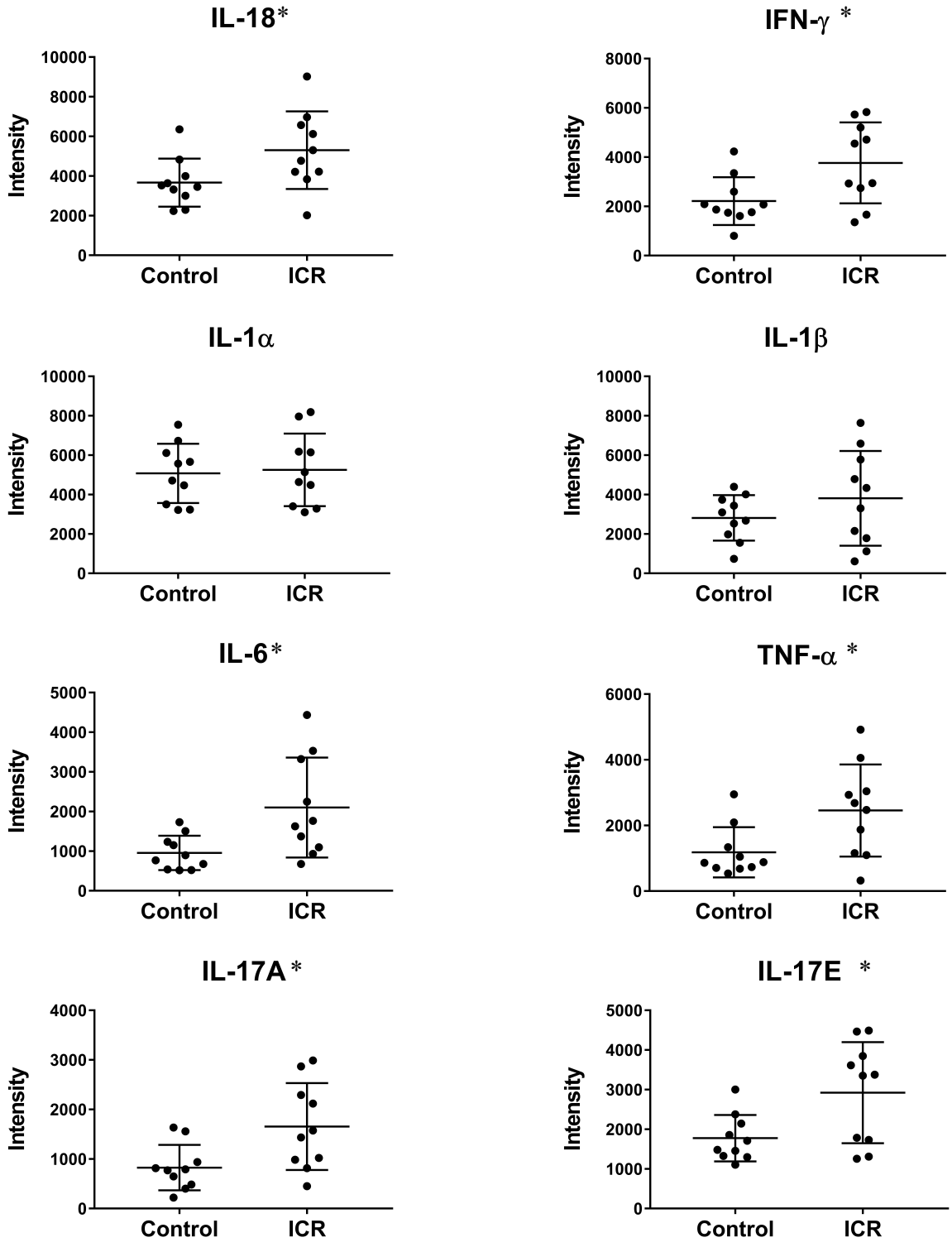
ICR = Idiopathic condylar resorption
* *p-value* < 0.05

Figure 15 (continued). Cytokine intensity in control versus ICR (Bar Graph)



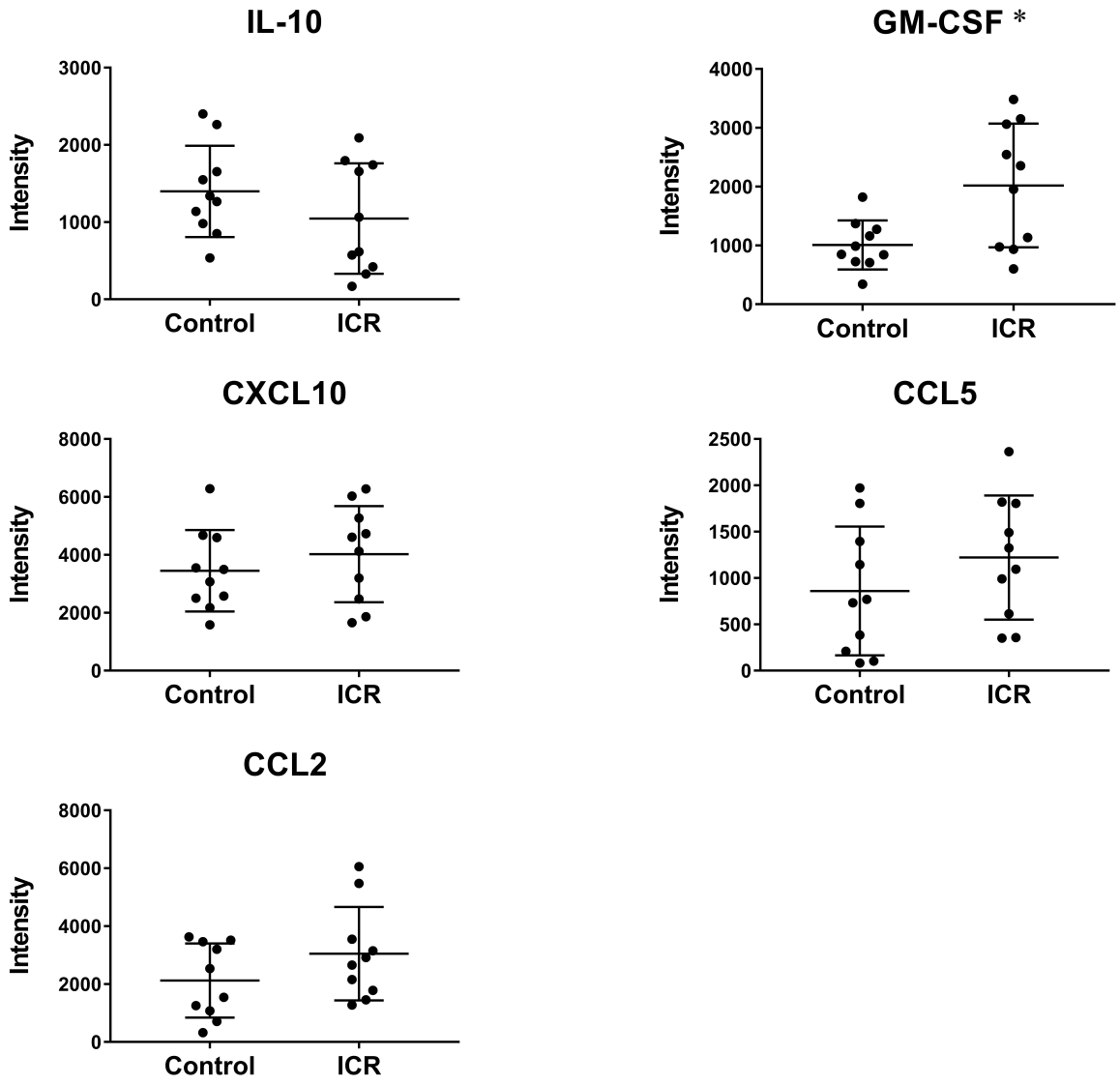
ICR = Idiopathic condylar resorption
* *p-value* < 0.05

Figure 16. Cytokine intensity in control versus ICR (Dot Plot)



ICR = Idiopathic condylar resorption
* *p-value* < 0.05

Figure 16 (continued). Cytokine intensity in control versus ICR (Dot Plot)



ICR = Idiopathic condylar resorption
* *p-value* < 0.05

Figure 17. Receiver operating characteristic (ROC) curves

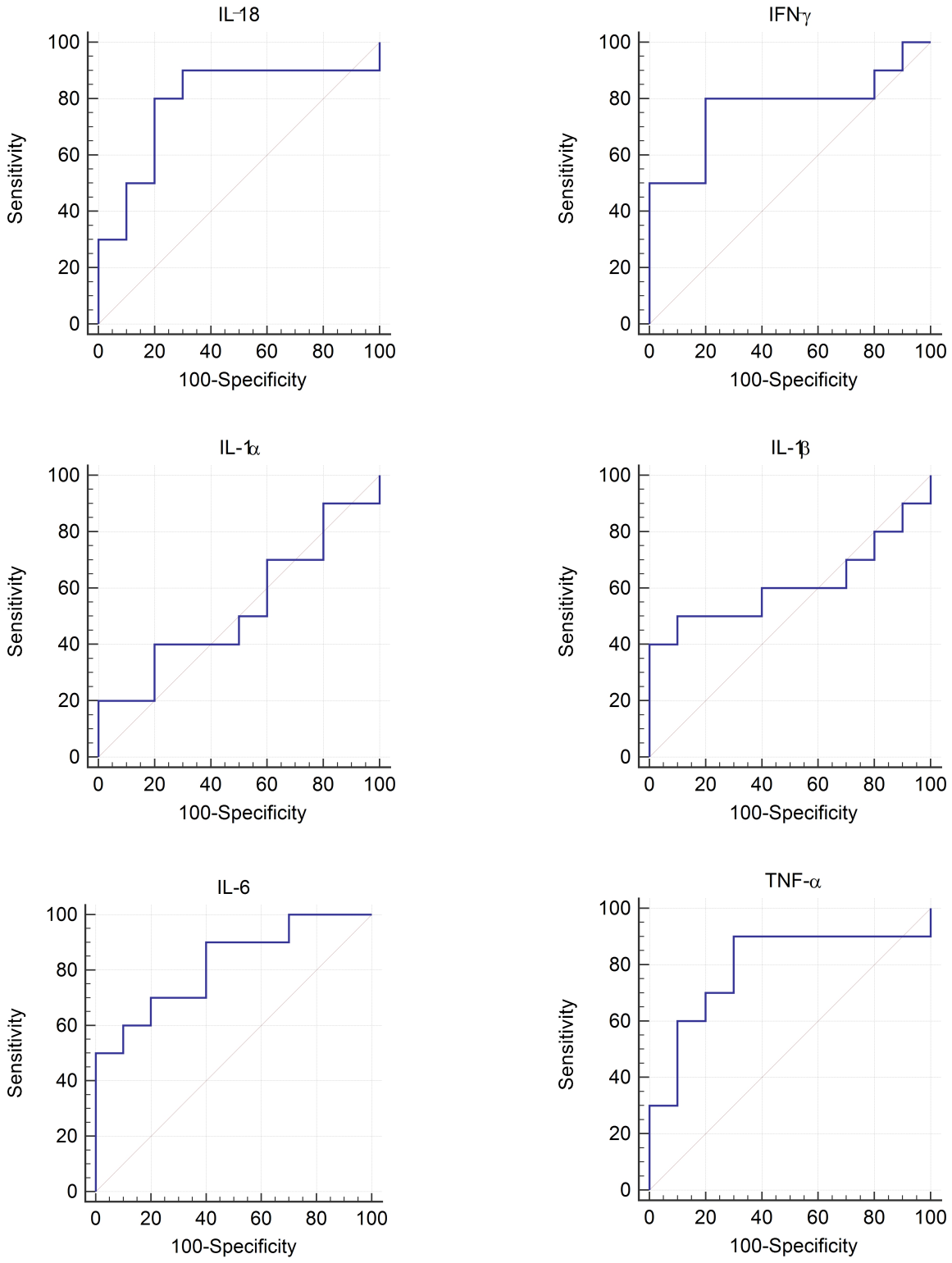


Figure 17 (continued). Receiver operating characteristic (ROC) curves

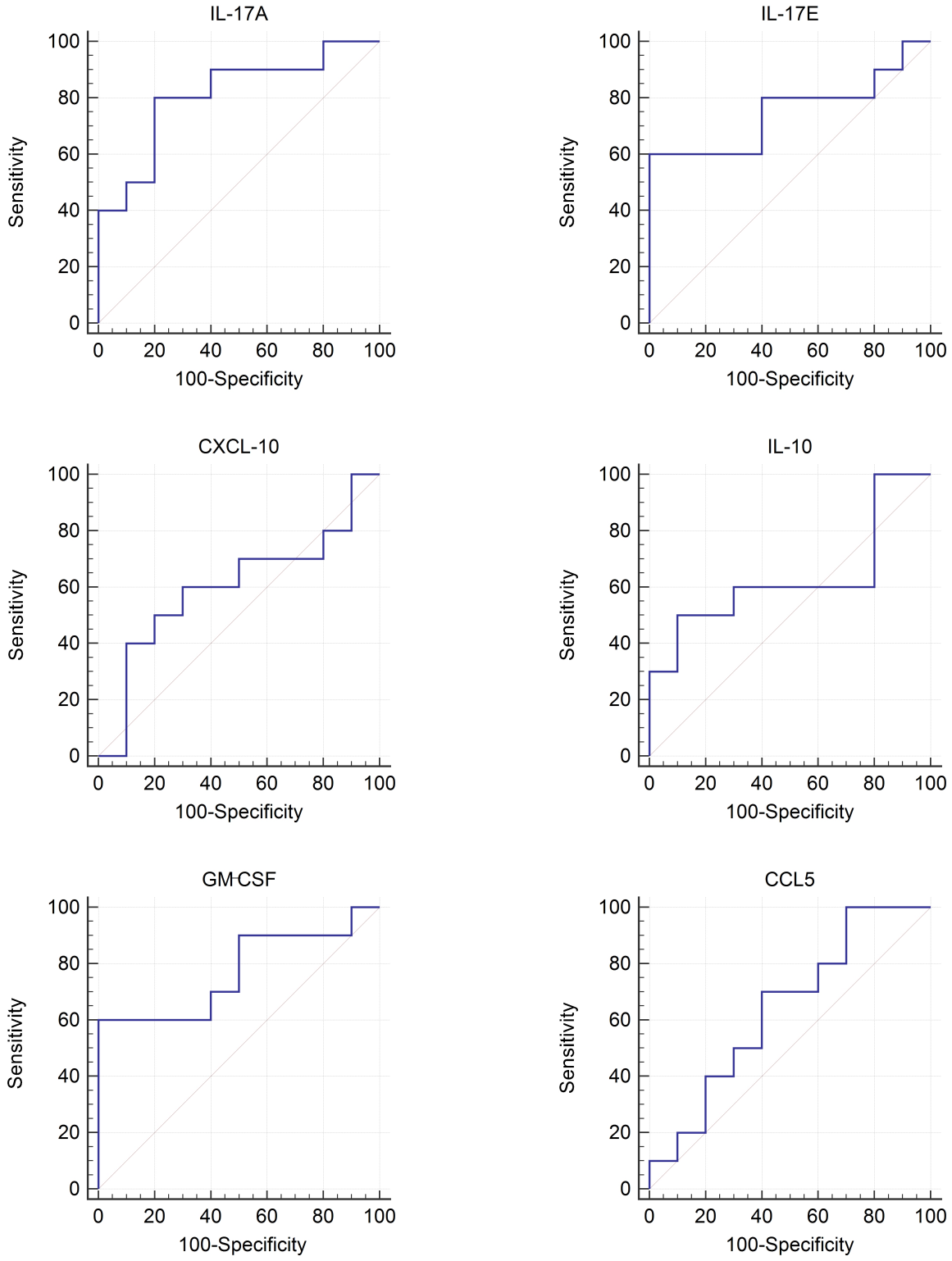
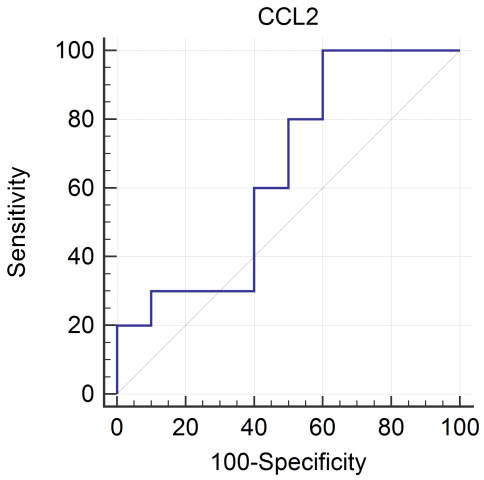


Figure 17 (continued). Receiver operating characteristic (ROC) curves



TABLES

Table 1. ILAR classification of JIA

Disease Type		Criteria
Systemic arthritis		• Arthritis in 1 or more joints either concurrently with or preceded by at least 2 weeks of fever, documented for a minimum of 3 days • Accompanied by at least 1 of the following: 1. Evanescent, erythematous rash 2. Generalized lymphadenopathy 3. Hepatomegaly and/or splenomegaly 4. Serositis *Exclusions: A, B, C, and D from the exclusion list below*
Oligoarthritis *2 Subtypes*	*Subtype #1:* Persistent oligoarthritis	• Arthritis involving 1-4 joints during the first 6 months • Affects a maximum of 4 during the disease course *Exclusions: A, B, C, D, and E from the exclusion list below*
	Subtype #2: Extended oligoarthritis	• Arthritis involving 1-4 joints during the first 6 months • Affects 5 or more joints after the first 6 months of the disease *Exclusions: A, B, C, D, and E from the exclusion list below*
Polyarthritis (RF negative)		• Tests negative for rheumatoid factor (RF) • Affects 5 or more joints during the first 6 months of the disease *Exclusions: A, B, C, D, and E from the exclusion list below*
Polyarthritis (RF positive)		• 2 or more RF tests taken at least 3 months apart are positive for RF • Affects 5 or more joints during the first 6 months of the disease *Exclusions: A, B, C, and E from the exclusion list below*
Psoriatic arthritis		• Arthritis and psoriasis present simultaneously, *or* • Arthritis and at least 2 of the following: 1. Dactylitis 2. Nail pitting (minimum of 2 pits on any nail) or onycholysis • Psoriasis seen in first degree relative *Exclusions: B, C, D, and E from the exclusion list below*
Enthesitis-related arthritis		• Arthritis and enthesitis present simultaneously, *or* • Arthritis or enthesitis with at least 2 of the following: 1. Sacroiliac joint tenderness with/without inflammatory lumbosacral pain 2. HLA-B27 antigen presence 3. Arthritis onset in male over 6 years old 4. Acute, symptomatic anterior uveitis • History of ankylosing spondylitis, enthesitis-related arthritis, sacroilitis with inflammatory bowel disease, Reiter's syndrome, or acute anterior uveitis in first-degree relative *Exclusions: A, D, and E from the exclusion list below*
Undifferentiated arthritis		• Arthritis that does not fulfill the criteria for any category above, *or* arthritis that fulfills the criteria for 2 or more of the categories above

Table 2. Exclusion criteria of the ILAR classification of JIA

Exclusion	Description
A	Psoriasis *or* a history of psoriasis in the patient or a first degree relative
B	Arthritis in HLA-B27 positive male with onset after 6 years of age
C	Ankylosing spondylitis, enthesitis-related arthritis, sacroilitis with inflammatory bowel disease, Reiter's syndrome, or acute anterior uveitis, *or* a history of one of these conditions in a first degree relative
D	Presence of IgM rheumatoid factor on 2 or more occasions at least 3 months apart
E	Presence of systemic JIA

Table 3. Mean intensity and standard deviation

Cytokine		Mean Intensity	Std Dev	Fold Change	*p*-value
IL-18	Control	3666.98	1211.48	1.45	0.0371*
	ICR	5305.16	1956.08		
IFN-γ	Control	2213.46	968.96	1.70	0.0192*
	ICR	3765.52	1643.97		
IL-1α	Control	5075.80	1502.68	1.03	0.8161
	ICR	5253.14	1840.96		
IL-1β	Control	2816.48	1153.34	1.35	0.2539
	ICR	3809.94	2403.12		
IL-6	Control	954.53	433.41	2.20	0.0140*
	ICR	2101.10	1259.86		
TNF-α	Control	1183.66	765.24	2.07	0.0214*
	ICR	2455.30	1401.04		
IL-17A	Control	826.63	459.94	2.00	0.0165*
	ICR	1655.03	877.95		
IL-17E	Control	1776.50	585.02	1.65	0.0187*
	ICR	2923.61	1275.65		
IL-10	Control	1397.99	591.48	0.75	0.2453
	ICR	1045.76	714.33		
GM-CSF	Control	1008.56	416.60	2.00	0.0112*
	ICR	2019.57	1051.43		
CXCL10	Control	3451.06	1404.66	1.17	0.4160
	ICR	4023.94	1661.58		
CCL5	Control	859.97	695.15	1.42	0.2523
	ICR	1220.85	669.17		
CCL2	Control	2125.46	1280.41	1.43	0.1740
	ICR	3047.65	1614.10		

**p-value <0.05*

Table 4. Sensitivity, specificity, criterion, and area under the curve (AUC) of receiver operating characteristic (ROC)

Cytokine	Sensitivity	Specificity	Criterion	AUC
IL-18	80%	80%	> 3992.8	0.79
IFN-γ	80%	80%	> 2595.68	0.77
IL-1α	40%	80%	> 6115.632	0.53
IL-1β	50%	90%	> 4012.184	0.61
IL-6	90%	60%	> 896.473	0.82
TNF-α	90%	70%	> 1049.562	0.79
IL-17A	80%	80%	> 937.55	0.81
IL-17E	60%	100%	> 3003.607	0.75
IL-10	50%	90%	< 616.9435	0.63
GM-CSF	60%	100%	> 1822.942	0.77
CXCL10	50%	80%	> 4596.075	0.60
CCL5	100%	30%	> 209.8515	0.64
CCL2	100%	40%	> 1255.374	0.65

AUC = area under the ROC curve

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