DETERMINATION OF THE PRESENCE OR ABSENCE OF ESTROGEN RECEPTORS IN THE HUMAN TEMPOROMANDIBULAR JOINT COMPLEX

by

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July, 1989

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Oral and Maxillofacial Surgery

SHORT TITLE

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ABSTRACT

There is a reported predominance of women who seek and accept treatment for symptoms related to temporomandibular joint (TMJ) pathosis. Previous studies have attempted to explain this observation on the basis of a sexual dimorphism of estrogen receptors (ERs) in the TMJ complex. The purpose of this study was to examine human TMJ complex tissue samples for the presence of ERs.

The subjects were 9 female patients, 16 to 30 years of age, diagnosed as having an internal derangement of the TMJ, and who were treated surgically. Biochemical ER assays were performed on the TMJ complex tissue samples using 17β-I^{125}-Estradiol. The tissue estradiol binding capacity was calculated using multiple point Scatchard plot analysis on the tissue samples and positive controls (rabbit uterus).

Results demonstrated ERs in the positive controls and the absence of ERs in tissue from the TMJ complexes of the subjects. It was concluded that there is no evidence to support a direct influence of estrogen on the tissue of the TMJ complex in humans.

RESUME

Selon la documentation relative aux symptômes intéressant l'articulation temporo-mandibulaire (a.t.m.), un plus grand nombre de femmes que d'hommes se plaindraient de troubles à ce niveau. Dans certaines études, les auteurs tentent d'expliquer cette constatation en invoquant le dimorphisme sexuel des récepteurs oestrogéniques (r.oe.) de la région de l'a.t.m. Cette étude vise donc à examiner des échantillons de tissus provenant de cette région afin d'y déceler, éventuellement, la présence de r.oe.

La population étudiée regroupe 9 patientes âgées de 16 à 30 ans chez qui on a diagnostiqué un dérèglement interne de l'a.t.m. et que l'on a opérées. On a effectué les dosages des r.oe. sur des échantillons de tissus prélevés dans la région d l'a.t.m. à l'aide du 17β-I^{125}-Estradiol. On a mesuré a capacité de liaison de l'estradiol aux tissue à l'aide de l'analyse graphique à point multiple de Scatchard sur les échantillons de tissus et sur des témoins positifs (utérus de lapin).

L'étude a démontré la présence des r.oe. dans les témoins positifs et l'absence de r.oe. dans les tissus provenant de la région de l'a.t.m. des sujets. Les auteurs concluent à l'absence de preuves confirmant le rôle des oestrogènes dans les tissus de la région de l'a.t.m. chez l'être humain.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. Timothy W. Head, who has directed this study and has given me constant help, guidance and support.

I am also grateful to Dr. Kenneth C. Bentley and, again, to Dr. Timothy W. Head, who provided the subjects for this study from their private cases. Appreciation is extended to the Oral and Maxillofacial Surgery Residents, Dr. Ian Ross, Dr. Cameron Clokie and Dr. Giorgio Aiello, who provided excellent pre-operative and post-operative care to the patients in this study.

Special thanks are in order to Mrs. Clara Incze for the tremendous amount of work which she did in the performance of the estrogen receptor assays.

My sincere thanks to Miss Fiona Lees for the typing of this manuscript.

INTRODUCTION

The majority of patients presenting for treatment of temporomandibular joint (TMJ) dysfunction are female.[1-33] The prevalence of women seeking treatment for TMJ disorders may be a reflection of their predisposition to develop osteoarthritis and other musculoskeletal disorders.[34-38] Females may be predisposed to TMJ disorders because of biochemical or physiological processes that differ from their male counterparts.[1,39,40]

Sex differences in hormonal influences on TMJ tissues would be a physiological mechanism that could explain differences in the prevalence of TMJ disorders between the genders. A sexual dimorphism in the distribution of estrogen receptors in the TMJ complex of the baboon has been demonstrated.[39,40]

Estrogen receptors (ERs) have been identified in many different cell and tissue types.[41-53] Target cells must have ER in order to be influenced by estrogens.[51,54-59] The effects of estrogen on protein synthesis (both collagen and non-collagenous protein), inflammation and wound healing in target cells and tissues have been shown.[60-74] If ERs could be demonstrated in the human TMJ complex, a mechanism for the predilection of TMJ disorders in females could be hypothesized.

The hypothesis, that sex differences in the prevalence of TMJ disorders is due to differences in hormonal activities between males and females, requires further investigation. Ovarian hormones, estrogens and progestogens, affect only specific tissues called target tissues because only these tissues have specific receptors that will bind the respective hormone to initiate their actions.[75] Does the TMJ complex of the human contain specific receptors to estrogen? Is the human TMJ complex a target tissue for ovarian hormones? If the human TMJ complex is a target tissue for estrogen, a biochemical basis could be established for the sex differences in the prevalence of TMJ disorders.

This study dealt with all patients diagnosed as having an internal derangement of the TMJ and who were treated surgically by the Oral and Maxillofacial Surgery service at the Montreal General Hospital from May, 1988 to March, 1989. Tissue for the study was obtained from the nine female patients treated surgically during the study period. There were no male patients with internal derangements treated surgically during this study period.

The following terms will be used throughout this paper and are defined as follows:

Temporomandibular joint dysfunction/disorder syndrome. The disorder has generally been defined or identified in terms of a set of symptoms rather than its etiology or by specifying the exact diagnosis.[76] Costen's original description of the disorder included a broad spectrum of symptoms: impaired hearing, sensation of burning in the tongue and throat, stuffiness, earache, dryness of the mouth, dizziness, tinnitus, vertical and occipital headaches associated with bite overclosure due to loss of posterior teeth.[77]

The presently accepted symptoms include one or more of the following: (1) pain and tenderness in the region of the muscles of mastication and TMJ; (2) sounds during condylar movement; and (3) limitation of mandibular movement.[20,78-87]

Internal derangement (displacement of the disc) of the TMJ. This is the anterior displacement of the disc of the TMJ in the closed jaw position without regard to condylar position.[88]

Ovarian hormones. The two types of ovarian hormones are the estrogens and progestogens. Three estrogens are present in significant quantities in the plasma of the human female: β-estradiol, estrone, and estriol. β-estradiol is considered

to be the major estrogen because of its relative potency. The most important progestogen is progesterone.[89] The ovarian hormones are steroid hormones synthesized in the ovaries mainly from cholesterol derived from blood.[89,90]

Hormone receptor. This may be defined as a molecular entity consisting of a receptive site, an executive site and a coupling mechanism. A **receptive site** can be defined as the initial point of steroid interaction, that is, the level at which information in the hormone is transferred to the cell. The message of the hormone is transferred via a **coupling mechanism** to the **executive site.** The executive site receives information from the receptive site and transmits either an amplified or recorded message to various loci in he cell. The message activates metabolic machinery responsible for producing specific hormonal responses.[58]

The hormone receptor's binding activity must fulfill the following criteria: hormone specificity, tissue specificity, high affinity, saturability and it must elicit a biological response.[58,59,91-93]

Epidemiologic studies have demonstrated an occurrence of symptoms and signs of TMJ disorders of 32% (range 16-59), and 61% (range, 33-86), respectively.[1,21,94-106] Seventy-five to 85% of patients seeking treatment for TMJ disorders are female.[1-33] Not all individuals with signs and symptoms of

mandibular dysfunction have a need of treatment. It has been estimated that 20-25% of investigated populations have a need of treatment of their symptoms.[21] If a sex linked cause of TMJ dysfunction could be elucidated on a biochemical and physiological basis, a treatment rationale for a significant portion of the patient population could be developed. However, it is generally accepted that the etiology of functional disorders of the masticatory system is complex and multifactorial.[81,107-110]

REVIEW OF THE LITERATURE

In 1934 Costen described a clinical condition in which patients presented with symptoms consisting of ear and sinus pain, tinnitus, hearing impairment and headache. On examination, these patients demonstrated tenderness in the TMJ area and overclosure of the mandible due to loss of posterior teeth. The symptoms were attributed to irritation of the auriculotemporal and chorda tympani nerves because of erosion of the glenoid fossa by the condyle. The erosion was thought to be caused by an abnormal posterior positioning of the condyle due to overclosure of the mandible. This symptom complex became known as Costen's syndrome.[77] There is no anatomical basis for Costen's explanation of the syndrome which he described.[111-113]

There have been many names applied to symptom complexes arising from dysfunction of the stomotognathic system. The terms used were usually based on what was thought to be the etiologic factor of the disease process.

In 1956 Schwartz described the **TMJ pain-dysfunction syndrome.**[114] He believed the patients pain was due to a persistent pain-spasm cycle caused by incoordination and spasm of the masticatory muscles. This theory shifted from the previous purely mechanical explanation to a theory that also included a psychological component.

The **myofascial pain dysfunction (MPD) syndrome** was introduced by Laskin and co-workers in the 1960s.[85] Muscle spasm was thought to be the primary cause of the pain-dysfunction syndrome. Parafunctional habits, such as grinding of teeth and clenching of jaws, were thought to be important etiologic factors.

During the 1970s and 1980s, numerous terms were used to describe TMJ disorders, including: mandibular dysfunction[1,115-117] craniomandibular disorders,[118] TMJ and muscle dysfunction syndrome,[119] and craniomandibular dysfunction.[121] Farrar and McCarty renewed interest in a mechanical cause for the signs and symptoms of TMJ dysfunction with their description of internal derangements.[120] The variation of terminology reflects disagreement between clinicians on the etiologic factors and pathogenesis of TMJ disorders.

Patient Studies of TMJ Disorders

Studies of patients seeking treatment for TMJ disorders report that most patients are female (70-90%). Hankey (1956)[14] noted that women were affected three times as often as men. Zarb and Thompson (1970)[6] found that 80% of TMJ dysfunction patients were women. Agerberg and Carlsson (1975)[12] reviewed 82 previous patients and found that 88% were women. Bultler et al (1985)[19] suggested that TMJ syndrome could be sex linked after a review of 56 patients revealed an 84% predominance of

women. Weinberg and Lager (1980)[7] reported on 90 clinic patients and 48 private patients. They found 8:1 and 2.7:1 ratios respectively of women to men. A recent study by Lundeen et al (1988)[23] showed an 80% prevalence of women in a group of 153 TMJ patients. There are many other reports that support these findings.[1-5,8-11,13,15-18,20-22,24-33]

Population Studies of TMJ Disorders

In the 1970s epidemiologic investigations of mandibular dysfunction were carried out either on complete populations[105,116,122-124] or on selected samples in accordance with statistically accepted principles[94,125,126] and with the use of well-defined and comparable diagnostic criteria. These studies were in contrast to earlier studies that used only patient material or violated the principles of pure epidemiologic studies.[2,20,22,127,128]

Helkimo (1972, 1974)[106,122] studied symptoms of mandibular dysfunction in 321 Lapps in northern Finland. He developed an index for dysfunction of the masticatory system. The index system is based on information furnished by the person examined (anamnestic dysfunction index) and on the symptoms and signs found at the clinical examination (clinical dysfunction index).[116] Helkimo concluded that the differences in the frequency of symptoms and signs of dysfunction with respect to sex were small and few. Neither the anamnestic nor the clinical dysfunction index differed with sex.[106]

Agerberg and Carlsson (1972)[94] reported on a random sample of every 35th individual, aged 15-74 years, residing in the Swedish city of Umea. Pain and symptoms of dysfunction of the masticatory system were relatively common and the sex distribution was more even than in clinical studies on record.

Agerberg and Osterberg (1974)[126] investigated 194 persons, or about 5% of the population, of 70-year-old inhabitants of Gothenburg. The investigation showed no appreciable difference between men and women in the frequency of different symptoms of pain and dysfunction of the masticatory system.

Agerberg and Carlsson (1975)[12] confirmed that women are overrepresented in clinical material. The randomly selected population sample demonstrated fairly equal sex distribution of mandibular dysfunction. These findings support Helkimo's conclusion that there is no difference in the prevalence of dysfunction between men and women in randomized populations.[4]

Few epidemiologic studies had been carried out on children and adolescents until the 1980s. Egermark-Eriksson et al (1981)[97] studied 402 Swedish children aged 7 to 15 years. They reported practically no sex differences in the occurence of clinical signs of dysfunction. Nilner (1983)[129,130] investigated 749 randomly selected subjects between 7-18 years of age. She stated that there was no statistically significant

difference in symptoms between boys and girls, when the presence of at least one symptom was considered. Ogura et al (1985)[131] studied 2,198 adolescents and reported no significant sex differences in TMJ sounds, pain, and limitation of opening. The investigation by Kirveskari et al (1986)[132] of 378 children aged 5, 10 or 15 years and living in southwestern Finland revealed no differences in the symptoms and signs of mandibular dysfunction between the sexes. One hundred and fifty-six Finnish children were studied by Kononen et al (1987)[133] in accordance with Helkimo's mandibular dysfunction index. They reported no significant difference between the sexes with respect to subjective symptoms and signs of craniomandibular disorders. Reports by de Boever and van den Berghe (1987),[134] Ohno et al (1988)[135] and Morawa et al (1985)[136] support the findings of the other investigators with respect to there being no significant sex differences in symptoms and signs of mandibular dysfunction in children and adolescents.

Kampe (1983)[137] reported on 125 individuals with intact dentitions and 163 individuals with restored dentitions. He found no statistically significant sex difference in either group. Sakurai et al (1988)[138] surveyed 220 completely edentulous patients undergoing routine examinations and treatment at an undergraduate dental clinic. They were not patients currently being treated for TMJ disorders. He reported no difference in the incidence of TMJ dysfunction symptoms between the sexes.

In contrast to population studies reporting no sex differences in the prevalence of TMJ disorders, many other population studies have found significant sex differences in symptoms and signs of mandibular dysfunction. Although significant, these differences have not been as great as in patient surveys.

A higher frequency of clinical signs of TMJ disorders have been reported in women. In many recent population studies, females have demonstrated a significantly higher frequency of TMJ noises.[1,15,94,97,102,130,139-147] Women more often display a smaller vertical opening or a decreased range of motion than men.[3,94,105,126,144,148-151] A female predominance in tenderness to palpation of the muscles of mastication and TMJ is often reported.[1,100,105,130,140,142,147]

Symptoms of TMJ dysfunction have also been reported to be more common in women as determined in epidemiologic population studies. Headache has often been found to be associated with signs and symptoms of mandibular dysfunction and it has been proposed that headache is one of the symptoms of mandibular dysfunction.[117,152-155] Although recurrent headache is more common among women,[3,94,105,130,142,143,147,156,157] it is a complex symptom with a variety of origins[158,159] and different forms of headache may occur in the same person.[160,161] In the

studies linking headache with mandibular dysfunction, no attempt was made to differentiate between different forms of headache in these studies.

In a number of studies, females reported tiredness in the jaws more commonly than males.[105,143,147,157] An inter-relationship between fatigue, muscular tenderness and headache has also been suggested in several studies.[160-163]

Helkimo's conclusion that there are no great differences in the frequency of mandibular dysfunction between men and women in the general population[21] is not supported by the studies cited above. His impression was based on three studies of nonpatients.[94,106,126] These population studies may not be comparable to the current population for various reasons such as the design of his studies, age and sex of the subjects involved, and differences in dental status.[1] Because of these defects, his conclusions cannot be applied to general populations.[1,139,142,164,165]

Magnusson and Carlsson (1978)[3] and Szentpetery et al (1986),[142] using Helkimo's indices of mandibular dysfunction, found a female predominance. Solberg et al (1979),[1] Gross and Gale (1983),[139] Rieder et al (1983),[140] Gazit et al (1984),[100] and Rugh and Solberg (1985),[166] in their population studies, found that women had a higher prevalence of mandibular dysfunction.

The population studies cited above have found significant differences in the prevalence of mandibular dysfunction between males and females. These findings reinforce the observation of a female predominance of TMJ dysfunction patients. However, the sex differences observed in population studies are not as dramatic as those observed in patient studies.[142] The different sex ratios in patient and population studies may be the result of several psychological and social influences.[8]

Autopsy Studies of TMJ Disorders

Several post-mortem examinations of TMJs have revealed a higher incidence of degenerative changes in women. Macalister (1954)[167] histologically examined 69 joints from subjects, aged 16 to 86 years, and found microscopic changes in 60. The most advanced changes were found in women. Blackwood (1963)[37] also found an unequal distribution of arthritic changes in the TMJ with a female predominance of degenerative changes. Oberg et al (1971)[34] examined 115 joints macroscopically at autopsy and found arthritic changes in 31% of the females and 15% of the males. Solberg et al (1985)[168] examined 96 TMJs of young adults at autopsy. They observed a greater frequency and severity of articular disc displacement in the TMJs of female specimens.

The incidence of acute conditions involving arthritis and general muscular skeletal disorders is observed at a ratio of 6:4 (women to men).[1] Female predisposition to degenerative

changes is not unique to the TMJ. Jorring (1980)[38] examined 6,321 patients undergoing radiographic examination of the colon. He found that above 60 years of age severe osteoarthritis of the hip was twice as common in women.

It has been suggested that gender differences in prevalence of TMJ disorders may be due to sex linked factors.[19,100] Solberg et al (1985)[168] suggested that women may be more susceptible to tissue alterations in the disc condyle complex. They observed contrasting adaptive responses in the TMJs of young men and women at autopsy.

Milam et al (1987)[40] suggested that estrogens may modulate certain metabolic events in the TMJ complex of the female baboon. They suggested that the human female predilection for certain pathologic states that affect the TMJ may have a pathophysiologic basis related to estrogenic effects.

Location of Estrogen Receptors

ERs are usually found in the cells of organs considered target tissues for estrogen. ERs have been identified in the uterus, vagina, corpus luteum, breast, brain and pituitary gland.[43] Recent research, however, has demonstrated nuclear uptake and retention of specific sex steroid hormones in cells of structures not classically considered to be target organs. These include the larynx,[53] gingiva,[42,50] liver, kidney,

adrenal, spleen,[218] cardiovascular system,[43] and central nervous system.[219,220] Target cells must have ERs in order to be influenced by estrogen.[51,54-59]

Aufdermorte et al (1986)[39] using autoradiographic techniques identified estradiol receptors in the TMJs of female baboons. In a continuation of the same investigation, Milam et al (1987)[40] failed to demonstrate ERs in the TMJ complex of male baboons. They suggested, that in humans, a hormonal influence on the TMJ complex of females is responsible for their predilection to TMJ disorders. This was based on a demonstrated sexual dimorphism in the distribution of ERs in the TMJ complex of the baboon. They noted that species differences in ER distribution made extrapolation to human populations only tentative.

Actions of Ovarian Hormones

Ovarian hormones regulate many metabolic, developmental and pathophysiologic events in various target cells and tissues.[40] Estrogen may serve to regulate a variety of metabolic processes in target cells and tissues including: inflammation and the immune response,[73,169-184,191-193,195] collagen and non-collagenous protein synthesis and degradation,[60-67,194,200] and bone and cartilage metabolism.[201-210]

The capacity of the female of various mammalian species, including man, to outperform the male when measured in terms of immune responsiveness has been documented many times.[73] Females are more resistant to bacterial and viral infections.[169] The incidence of autoimmune disease is higher in females.[170] Also, females reject allografts more often than males.[171] In experimental and natural conditions, the female may generate enhanced immunoglobulin production in all classes of antibodies.[172-176]

Changes in sex hormone levels exert a marked influence on immune responsiveness and stem cell differentiation: by increasing numbers of functioning cells, by promoting cellular differentiation, as well as by promoting cellular function via hormonal effects.[73] Androgens and estrogens produce lymphoid atrophy of both thymus and peripheral lymphoid organs.[177-180] Gonadectomy, both in the male and in the female, has been associated with lymphoid hyperplasia.[181] In vitro, estrogens suppress the acitivity of suppressor T-cells,[182] increase the number of macrophages in experimental granulomas and are powerful stimulants of macrophage phagocytic activity.[183] Progesterone in concentrations produced locally at the placental maternal interface has demonstrable immunosuppressive properties including anti-inflammatory and graft-sparing effects when administered locally in animals.[195]

Administration of estrogens can potentiate autoimmune diseases. Symptoms of rheumatoid arthritis are increased by the administration of estrogen.[170] In rats, a polyarthritis, which is radiographically similar to rheumatoid arthritis, can be induced by the systemic administration of peptidoglycan-polysaccharide fragments derived from cell walls of bacteria. Males are relatively resistant to streptococcal induced polyarthritis as compared to females. However, the administration of estradiol causes males to become as susceptible as females.[184]

There are many examples of sex hormones modifying the inflammatory process. A specific example is human gingiva. Biochemical studies have identified sex steroid receptors in gingiva.[42] There is a clinical correlation between pathological states of the periodontium and circulating levels of sex steroid hormone.[72] There is increased gingival inflammation in pubescent males and females.[185] The fluctuation of gingival exudation during the menstrual cycle was observed.[186,187] The exacerbation of gingival inflammation during pregnancy which regressed post- partum was noted.[188,196-199] Gingival changes were reported in females given birth control pills that closely resembled those observed during pregnancy.[189] The elevation of circulating progesterone in both males and females with periodontal disease was reported.[190]

Milam et al (1987)[40] suggested that estrogen enhancement of the immune response may contribute to both cartilage and bone destruction in certain degenerative, inflammatory processes that affect the TMJ.

Estrogen has a singificant effect on collagen synthesis and degradation. Kao et al (1964)[64] reported that estrogen played a specific role in collagen formation within the uterus. Woessner (1969)[67] concluded that estrogenic hormones inhibited the breakdown of collagen in the rat uterus. Henneman (1971)[62] observed that estradiol-17β administered to adult female guinea pigs produced an increase in the biosynthesis of both collagen and non-collagenous protein in metaphyseal bone and in the uterus. This increased synthesis seemed to be at the expense of reduced biosynthesis and increased degradation of collagen in the skin. Many other researchers have noted similar effects on collagen and non-collagenous protein metabolism caused by estrogen administration.[60,61,63,65,66,194,200]

Clinical studies suggest that estrogen is important for the preservation of mineralized bone. Postmenopausal diminution in estrogen metabolism appears to be the most important cause of osteoporosis.[201-205] It has also been noted that estrogens minimize bone loss[206-209] and that degenerative changes of bone will either slow or cease with the use of estrogens.[206-208,210]

A number of studies involving induction of cell proliferation, accurate remodelling and termination of new growth suggest that hormones may help to regulate wound healing.[68,69,211-214,216,217] Estrogens have been shown to potentiate wound healing.[211,212] Progesterone also accelerates vascularization.[215] Studies have shown estrogenic, progestenic, and androgenic potentiation of cutaneous healing rates with increased production of fibrinous substances and dilation of local blood vessels.[211-214] Ovarian hormones could effect the response of the tissues of the TMJ complex to injury through their effect on wound healing.

It is therefore possible that estrogens, through their influence on wound healing, the inflammatory and immune processes, collagen and non-collagenous protein synthesis, and bone metabolism, may predispose women to TMJ disorders. Estrogens could modify the female response to macro-trauma and micro-trauma in such a way as to result in greater damage to the TMJ from these insults. The result of this would be a prevalence of women with TMJ disorders.

Identification of Estrogen Receptors

The era of emphasis on receptor-mediated actions of the steroid hormones began with the pioneering studies of Glascock and Hoekstra (1959),[222] Folca et al (1961)[221] and Jensen and Jacobsen (1962).[226] These investigators demonstrated selective concentration and retention of ^{3}H-labeled estrogens in tissues

known to be targets for estrogen action as established by previously described physiological effects. It was demonstrated that this concentration was due to the presence of a specific protein, the "estrogen receptor", that binds estrogens with a high affinity.[223] In order to observe a response to estrogenic stimulation or deprivation in a tissue, ER must be present.[51,54-59]

Five specific criteria must be fulfilled before a protein can be designated as a receptor.[58,91,92] The binding between the receptor and hormone must demonstrate hormone specificity, tissue specificity, high binding affinity, saturability, and a biological response.

Hormone specificity implies that only those steroids or analogues having similar biological capacity have been found to compete for a particular binding protein.[58] Using ER as an example, it can be demonstrated that ER binds estradiol but not testosterone, a molecule that differs from estradiol by the presence of an additional methyl group and minor changes in the A-ring structure.[91,92]

Tissue specificity implies that hormonal steroids affect only cells of specialized tissues. The feature that distinguishes target tissues from non-target tissues is the presence of receptors in target tissues.[58]

Steroid hormones demonstrate **high affinity binding** by binding to specific receptors at low concentrations.[91,92] Underwood (1983)[59] described three classes of steroid binding activity which he designated types I, II and III. The Type I binding site for estrogens is the classical ER and is present only in estrogen responsive cells. It is characterized by a high affinity and specificity for estrogenic steroids. Type II binding sites are specific for estrogens but have a lower affinity than has classical ER. The biological and clinical significance of these low affinity sites awaits evaluation. Type II binding sites are ubiquitous and have a very low affinity and specificity for steroid hormones. However, Type III binding sites do constitute a very substantial binding capacity in tissues and body fluids.

Another criterion required to designate a protein as a receptor is **saturability**. Since graded metabolic responses to steroids occur within the range of physiological hormone concentrations, receptors should become saturated over the same range.[58] For example, the dissociation constant (K_d) for the binding between ER from human breast cancer and estradiol is on the order of 1×10^{-10} M, which means that the half-saturation point is reached at normal physiological concentrations of estradiol. Thus, small changes in the physiological concentration of estradiol modulate the hormone-induced response in the tissue.[91,92]

An example of a **biological response** due to a hormone binding to a receptor can be demonstrated in the rat uterus. Within one day after stimulation of uterine tissue of the immature rat with estradiol, the gain in uterine weight characteristic of maturation as well as synthesis of specific estrogen-dependent proteins such as progesterone receptors has taken place.[225,227]

Structure of Estrogen Receptors

Receptors are thermolabile proteins.[228,229] Evidence suggesting that receptors for steroid hormones are proteins includes: their stereospecificity,[230] their size as determined by gel filtration chromatography[231-233] and sucrose gradient analysis,[55,223,232] their limited number of binding sites,[230,232] their sensitivity to heat and sulfhydryl reactive reagents,[230,232] and their sensitivity to proteases but not nucleases.[230,232,233]

Subcellular Location of Estrogen Receptors

The subcellular location of steroid receptors has been studied. It was first assumed that native receptors were extranuclear proteins, and that after hormone-receptor complexes were formed, translocation from the cytoplasm to the nucleus occurred, followed by firm binding to the the nucleus.[232-235] Receptor transformation from the 8S to the 5S form and the nuclear translocation were thought to be temperature dependent phenomena.[55,232,236] The hypothesis was

that unoccupied receptors were localized at extranuclear cellular sites (represented by cytosol) until forming a hormone-receptor complex upon hormone exposure. The hormone acted to induce receptor transformation to the active biochemical form that was capable of penetrating the nuclei.[232,237] Recent studies making use of various experimental approaches, including immunohistochemical staining, have established that the native receptors for all types of steroid hormones reside predominantly within the nuclear compartment.[46,47,238-244]

Specific ERs exist as either "free" (unoccupied) or "filled" (occupied) receptors.[245-247] Free ERs are not bound to endogenous hormone and are easily extractable from tissue homogenates with buffers of low ionic strength.[91,92] Filled ERs are bound to endogenous estradiol and are extractable only with buffers of high ionic strength.[91,92] Because of the techniques used to prepare the tissue for analysis, free receptors are mainly detected in the cytosol and filled receptors are only found in the nuclear fraction.[248,249] Studies of the correlation between cytosolic and nuclear ER concentrations [250-252] indicate that an equilibrium may exist between the two forms of ER in vivo.[91,92]

Estrogen Receptor Assays

Hormone-responsive tissues contain receptor protein that constitutes .01% to .001% of total cellular protein.[227]

Traditional biochemical assays for ERs are based upon binding of radioactive ligand to receptor.[91,92] The recent development of monoclonal antibodies directed against estrogen[253] and progesterone[254] receptors has initiated new types of assay methods for receptors.[47,255,256] Monoclonal antibody techniques are based upon binding of antibody to the receptor protein. Ligand binding techniques detect free ER and PgR in tissue homogenates while monoclonal techniques detect both free and filled receptors.[91,92]

Initial **biochemical ligand binding assays** for detection of ER used ^{3}H estradiol to study tissue binding.[257-260] Hochberg (1979)[261] first synthesized the iodinated estradiol derivative 17β-[16-I-125]-estradiol. This derivative was successfully used to bind ERs with high affinity and specificity. Its binding characteristics, which include greater sensitivity with small tissue samples and technical advantages in determining radiation from a gamma emitter as compared to a beta emitter, make the iodinated derivative the compound of choice over the tritiated derivative for estrogen receptor binding studies.[262]

Ligand binding assays for determination of cytosolic ER and PgR include the following steps: homogenization of tissue, preparation of the cytosol, binding of radioactive hormone to cytosol proteins, incubation of the sample until equilibrium is achieved, separation of free and bound hormone, resolution of specific and non-specific binding, and estimation of the number of hormone molecules bound.[91,92]

The cytoplasmic estrogen receptor is highly temperature labile.[228,229] Immediately after excision, tissue samples should be cooled, preferably in liquid nitrogen.[263] The loss of binding activity is partially due to proteolytic cleavage of the receptor by endogenous enzymes present in the tissue sample. Some of these proteases are Ca^{2+} dependent, therefore removal of endogenous calcium by chelation with EDTA will stabilize the receptor.[264] The additon of monothioglycerol also protects the labile receptor and improves the sensitivity of the assay.[265] Homogenization of the nitrogen-cooled tissue blocks and lyophilization of the pulverized tissue allows storage for several months at 0-4°C without a decrease in ER binding sites.[266] The use of lyophilized tissue aliquots has been recommended for intra- and inter-laboratory quality control studies of steroid receptor measurements.[266-269]

Separation of bound and free hormone following incubation can be accomplished by several different techniques. Steroid receptor determinations can be divided into quantitative and qualitative assays. Quantitative assays use dextran-coated charcoal,[270] hydroxyapatite,[271] or protamine sulfate.[272] Receptor assays that also provide a qualitative characterization include: sucrose gradient analysis,[273] agar gel electrophoresis,[280] or column chromatography.[281] All assays are based upon the fact that the hormone-ER complex is extremely stable. Therefore, unbound estradiol can be eliminated at 0-4°C without significant dissociation of the complex.[274]

The most commonly used method for separation of the bound and free hormone is the dextran-coated charcoal (DCC) method. The technique was first introduced by Korenman and Dukes (1970)[270] and was modified by several other investigators.[275-278] Aliquots of cytosol are placed in two series of centrifuge tubes. To one series increasing doses of radioactive estradiol are added. The second series contains the same concentrations of labeled hormone plus a hundred fold excess of unlabeled competitors such as 17β-estradiol itself or the synthetic estrogen, diethylstilbestrol, to determine non-specific binding. The tubes are incubated at 0-4°C for at least 4 hours, sufficient time to achieve more than 95% maximal binding. Subsequently, a charcoal-dextran suspension is added to remove unbound hormone from the estradiol-cytosol mixture by adsorption on the dextran-coated charcoal. Dextran is a long, fibrous compound that coats charcoal particles and prevents the adsorption of large protein molecules by the charcoal but allows small unbound steroid molecules to be adsorbed. The difference in receptor-bound hormone remaining in solution in the absence and in the presence of the competitor gives an indication of the specific or saturable binding of hormone to the receptor. Knowing the ratio of bound to free steroid in the original mixture one can calculate the number of receptor binding sites and the equilibrium binding constant. The multipoint titration analysis employing the Scatchard plot method is most often used for the interpretation of the data.[279]

While biochemical assays for ER may indicate the presence of a steroid binding capacity per unit weight of tissue protein, they cannot identify the cellular origin of the receptor protein. Another drawback of the biochemical assay is the large amount of tissue (0.2-0.5 g) needed for accurate determination of binding.[274] Because of these disadvantages several laboratories have undertaken the developemnt of immunologic and cytochemical techniques of ER determination.

The **labeled-ligand method** is one cytochemical method of demonstrating ER. The earliest morphological demonstration of estrogen binding depended upon the autoradiographic detection of tritium in sections cut from fresh tissue that had first been incubated in tritium-labeled estradiol.[282] However, the quantitative difference between total binding (with tritiated estradiol alone) and nonspecific binding (with tritiated estradiol in the presence of an excess of diethylstilboestrol) is difficult to assess in histological preparations without resorting to quantitative methods.[59] In order to reduce the number of technical steps in the morphological assay, direct fluorochrome or enzyme labeled-ligand procedures have gained recent popularity.[283-285] Lee (1978,1979)[284,286] noted a lack of correlation between his cytochemical method and the biochemical assay. These disparate results of biochemical and morphologic techniques are further compounded by an apparent lack of agreement between the individual morphologic methods.[274] The relative binding affinity of the

fluoresceinated estrogens to Type II and Type III binding sites may be higher than to classical Type I binding sites.[59] At the concentrations used, the fluorescein-labeled estrogens may attach to the lower affinity Type II and Type III binding sites and tissue that is ER-negative, as measured by biochemical assay, will appear positive with morphologic methods.[274]

Immunological methods of detection of ER and PgR include **immunohistochemical analysis (ICA)** and **enzyme-immunochemical analysis (EIA)**. Specific monoclonal antibodies to ER protein have been prepared[253] which can be used for biochemical[293] and cytochemical[47] ER determination. Non-immunological assays are based on the evaluation of the binding capacity of tissue cytosols for radiolabeled estrogens and therefore cannot reliably measure receptors in the presence of high concentrations of endogenous estrogens or anti-estrogens.[256] Also, the binding capacity of the receptor is diminished by exposure to traces of heavy metal ions, or by degradation of the labile receptor protein during storage and processing of the tissue specimen.[287] These difficulties have recently been overcome by the introduction of immunoassays. These assays, which are based on monoclonal antibodies to human tumor ER produced by Greene et al (1980),[253] use the direct antigenic recognition of the receptor molecules. The antibodies recognize the ER independent of the presence or absence of estradiol in the binding site.[256]

The **immunohistochemical assay method** detects receptors in lightly fixed frozen tissue sections. Monoclonal antibodies raised in the rat against human receptor are applied to tissue sections and using the peroxidase-antiperoxidase method for immunocytochemical staining, the binding of the monoclonal antibodies can be visualized.[91,92] Excellent correlations between the biochemical assay results and the ER-ICA assays results have been found in the studies published to date.[48, 288,290,292] A major disadvantage of the ICA assay method is that unless advanced image-analyzing systems are employed, results are semi-quantitative.[91,92]

The **enzyme-immunochemical assay method** is an immuno-biochemical method for quantitation of receptors. In the EIA assay the primary monoclonal antibody is bound to a polysterene bead, while peroxidase is coupled to the second monoclonal antibody, as in the case with the ICA assays. The final signal that is measured and which represents the number of receptor molecules in a given sample, is the intensity of color developed.[91,92] This is also analogous to the method used in the ICA assay. Excellent correlations between the ER-EIA and ER-DCC assay methods have been reported in multicenter European[256] as well as American[289] studies. ER-EIA assay requires only one-twelfth the volume of cytosol necessary for an ER-DCC multipoint titration analysis.[91,92] The EIA method requiring much less tissue can be used in fine needle tissue aspirates.[291]

Subcellular Mechanism of Induction of Biological Response Associated with Estrogen Receptors

The relationship between occupied steroid hormone receptors and the induction of biological response, as defined by synthesis of a specific protein, has been examined by a number of laboratories. Simple linear[294-297] or exponential[298,299] responses between receptor occupancy and protein induction has been observed. There may be at least three different relationships between receptor occupancy (level and time) and biologic response. The response may be proportionate to the degree of maximum occupancy.[297,300] The response may be proportionate to the degree of receptor retention and residency time.[301] The maximal response may be achieved at low levels of occupancy.[298,302]

Mueller et al (1958)[303] first proposed that the steroid hormones control production of nucleic acid templates and hence, gene expression. Studies demonstrating a nuclear location of the receptor and stimulation of RNA synthesis support the concept that the primary site of steroid hormone action might be within the cellular genome.[55,232,233,236,237] Steroid hormone receptors alter nuclear gene transcription, leading to the production of all classes of RNA before regulating cytoplasmic protein synthesis.[233]

The acceptor site hypothesis states that steroid receptors interact with one or more classes of a limited number of specific nuclear binding sites.[235,304] It is now widely accepted that there are specific chromatin-localized acceptor sites for steroid-receptor complexes.[305-308] The steroid hormone receptors represent the first gene-regulatory proteins described in eukaryotic systems.[309,310]

MATERIALS AND METHODS

Subjects

The population defined for the purpose of this study included all patients requiring open TMJ arthrotomy who were treated by the Oral and Maxillofacial Surgery Service at the Montreal General Hospital from May, 1988 to March, 1989. Tissue was obtained from the TMJ complexes of 9 female patients. The patients' ages ranged from 16 to 30 years and all were diagnosed as having TMJ internal derangement (Table 1).

There were no male patients treated surgically for TMJ disorders during the study period. Tissue was obtained from diseased TMJs only as ethical considerations prevented the harvesting of tissue samples from normal human TMJs.

Procedures

The subjects selected for this study were patients who accepted surgical treatment for the management of their TMJ disorders. The principal criteria used to determine suitability for surgery were pain or limitation of function of the TMJ that severely limited the patients' daily activities. All patients had initially failed conservative therapy consisting of all or some of the following treatments: counselling, behaviour modification, analgesics, anti-inflammatories, muscle relaxants, physiotherapy and occlusal

splint therapy. Furthermore, all patients demonstrated intra-articular disease consisting of an internal derangement of the TMJ diagnosed on the basis of history, clinical examination and arthrographic findings.

The study was designed to determine the presence or absence of ERs in the tissue of human TMJs with internal derangements. The biochemical method selected to assay for ER limited the scope of the study. A minimum of 200 mg of tissue is preferred for the assay. The quantity of tissue required for the assay prevents the harvesting of tissue samples from normal TMJs. The study was therefore limited to tissue obtained from diseased TMJs at the time of surgery. All patients treated surgically during the study period were female.

Tissue assayed for ERs included: disc, capsule, posterior attachment and articular cartilage from the TMJ complex. No modifications of the routinely used surgical technique were required to obtain the tissue samples.

Surgical Technique

The surgical procedure used to treat the patients in this study was that described by Walker and Kalamchi (1987).[311] All surgical procedures were performed under general anaesthetic in the operating theatres at the Montreal General Hospital.

The patients were brought into the operating room and placed on the table in the supine position. General anaesthesia was induced and maintained by nasotrachial intubation. Dental arch bars were secured to the maxillary and mandibular teeth using 24 guage stainless steel circumdental wires. The patients were prepared and draped in routine fashion.

A preauricular incision was used to gain access to the TMJ. The wound was deepened to the temporalis fascia by blunt and sharp dissection and then extended inferiorly along the temporalis fascia to the zygomatic arch and upper extent of the TMJ lateral capsule. The capsule was cleanly exposed by blunt dissection in an inferior direction for 1.0 to 1.5 cm. A vertical incision was made through the capsule directly to the bone of the condylar neck and extended superiorly to the zygomatic arch. The upper limb of this incision remained just superficial to the disc and opening of the upper joint space was avoided at this point. The periosteum and capsular attachments were freed around the condylar neck, and the lower joint space was entered to expose the condyle. Dunn-Dautrey retractors (Walter Lorenz Surgical Instruments, Inc.) were used to protect the soft tissues about the condyle. Approximately 2-4 mm of bone and articular cartilage from the height of the condyle was excised, using a no. 701 crosscut fissue bur, under sterile saline irrigation. A portion of this specimen was sent

for pathological evaluation and the remainder was placed in a plastic container and stored immediately on dry ice. The condylar stump was smoothed with a bone file or round bur.

The upper joint space was opened via a horizontal incision between the disc and the zygomatic arch. The disc was carefully freed from its displaced position (usually, in an anterior and medial direction), and drawn over the condylar stump, ensuring that the posterior band of the disc covered the posterior edge of the condylar stump. A wedge of redundant disc and posterior attachment was excised including a portion of lateral joint capsule. Representative samples were sent for pathologic evaluation. The remaining tissue was placed in a plastic container and immediately stored on dry ice.

Using a no. 6 round bur, a hole was drilled through the posterior cortex of the condylar stump as far medially as possible and approximately 3 mm below the cut edge. The hole extended upward into the superior part of the stump. A second hole was drilled through the lateral cortex of the condylar stump approximately 3 mm below the cut dege, extending upward into the superior part of the stump. The disc was then drawn over the condylar stump and fixed securely by passing separate 2-0 polyester fiber sutures (Mersilene) through the two holes and then through the posterior band of the disc. As the disc was held tightly posteriorly and laterally, the two sutures were tied securely fixing the disc atop the condylar stump.

The jaw was then moved through various functional positions to ensure that the disc-condyle complex moved easily without obstruction. The wound was then closed in layers and a protective pressure dressing was placed.

Physiotherapy was begun immediately post-operatively and the patients were asked to wear elastics attached to the dental arch bars at night to hold the teeth in firm occlusion while the patients slept. Range of motion exercises were carried out for a period of one month to develop an interincisal clearance of at least 40 mm. The regime of daytime use of the jaw and nighttime immobilization in the correct occlusal position was continued for three months.

Estrogen Receptor Assay

The ER assay technique used was a biochemical analysis based on the binding of radioactively labelled estradiol to specific receptor sites in the tissue samples. The "Radio Receptor Assay Kit for the Quantitative Measurement of Estrogen Receptor in Tissue Cytosol" used for the study was provided by Diagnostic Systems Laboratories Inc. (DSL) (Catalog No. DSL 2800, Diagnostic Systems Laboratories Inc., P.O. Box 57946, Webster, Texas, 77598).

The DSL I^{125}-estrogen receptor assay kit provides materials for the quantitative measurement of ERs in tissue cytosol. The DSL I^{125}-ER assay is performed by incubating

varying quantities of I^{125}-estradiol with a constant amount of receptor protein (cytosol). The analysis is based on the establishment of the following equilibrium.

I^{125}-Estradiol + ER = I^{125}-Estradiol ER + I^{125}-Estradiol

The unbound I^{125}-estradiol is then adsorbed with the dextran-coated charcoal (DCC) and separated from the I^{125}-estradiol ER complex. Nonspecific binding is assessed by simultaneously incubating I^{125}-estradiol and cytosol with an excess of diethylstibestrol, a non-radioactive synthetic estrogen analog. Estrogen receptor numbers (binding capacity) are calculated using the multiple point Scatchard plot analysis.

The materials supplied in DSL's I^{125}-Estrogen Receptor Assay Kit include the following:

1. **Positive Control** Three vials containing estrogen receptor (rabbit uterus) positive control in buffered medium (lyophilized).
2. **Assay-Buffer** One bottle containing 125 ml of Tris HCL buffer, EDTA, glycerol and 0.1% sodium azide as a preservative.
3. **Monothioglycerol** One vial containing 1.0 ml of monothioglycerol.

4.	**Diethylstilbestrol**	One vial containing 1.5 ml of 50 µg/ml of diethylstilbestrol (DES) in Tris HCL buffer.
5.	**Separating Reagent**	One bottle containing 125 ml of activated dextran-coated charcoal (DCC) with a 0.1% sodium azide as a preservative in Tris HCL buffer.
6.	**I^{125}-Estradiol**	Six vials containing 4 ml of I^{125}-Estradiol in Tris HCL buffer with 0.1% sodium azide as a preservative. Tracer solutions of levels 1-6 are approximately 2.5, 1.25, 0.75, 0.38, 0.19, and 0.10 µCI/ml respectively.
7.	**BSA Standard**	One vial containing 1 ml solution of 0.1% (1 mg/ml) crystalline bovine serum albumin, fraction V, in Tris HCL buffer.

All materials must be stored at 2-8°C. The lyophilized controls must be reconstituted with 3.0 ml/vial of chilled deionized water just prior to the assay.

Materials Required for the Assay Not Supplied in the DSL Estrogen Receptor Assay Kit

1. 12 x 75 mm and 16 x 125 mm glass tubes.
2. 10, 50, 100, and 500 µg pipettes with disposable tips.
3. Refrigerator.

4. Centrifuge capable of > 1500 rpm (refrigerated).
5. Centrifuge capable of > 45000 rpm.
6. Tissue homogenizer.
7. -70^{o}C freezer.
8. Gamma counter.
9. Vortex mixer.
10. Test tube racks.
11. Normal saline.
12. Cold room.
13. Magnetic stirrer and stir bar.
14. Forceps.
15. Petri dish.
16. Razor blade.
17. Spectrophotometer.
18. 0.5% copper sulfate.
19. 2% sodium carbonate.
20. 2% sodium tartrate.
21. 0.1 N sodium hydroxide.
22. 2 N phenol (Folin-Ciocalteau) reagent.

Specimen Collection and Preparation

A. Specimen collection

Immediately after harvesting the tissue sample, efforts were directed to prevent thermal denaturation of the receptors. The sample was rinsed with normal saline, placed in a sealed plastic container and the sample was carried to the laboratory on dry ice. The tissue was stored in the freezer at -70^{o}C until the assay was performed.

B. **Cytosol preparation**

1. The monothioglycerol-buffer (MTG-buffer) was prepared by adding 1 part monothioglycerol to 1000 parts of the assay buffer. Four ml of MTG-buffer was required for each ER assay and 10 ml of MTG-buffer was required for each protein assay. The MTG-buffer was kept chilled on ice and fresh buffer was prepared for each run.

2. Immediately before the assay, the tissue sample was removed from -70^{o}C storage. On a glassine weighing paper cooled in dry ice, the tissue sample was weighed and then placed directly into an appropriately labeled 16 x 125 mm tube.

3. Four ml of pre-chilled MTG-buffer was added to the labeled tube and the tube was returned to the ice bath. An approximate ratio of 1:10 (w/v) was maintained for the tissue weight/MTG-buffer volume. This will usually result in a cytosol protein concentration of 2-5 mg/ml.

4. The mixture of tissue and MTG-buffer was homogenized while the tube remained immersed in an ice water. It was homogenized in 3 bursts of 5 seconds in duration, allowing 15 seconds of cooling in ice between bursts (Polytron PT 10 homogenizer with the probe generator set at half speed).

C. **Centrifugation**

The centrifuge rotor was precooled for at least a 1/2 hour before use. The homogenate was quickly vortexed and decanted into a labeled polycarbonate tube that was immersed in an ice bath. The tube was balanced and capped. The homogenate was centrifuged at 45000 rpm for 1 hour at 4°C.

D. **Supernatant (cytosol) transfer**

The supernatant was transferred from the polycarbonate tube to a labeled 16 x 125 mm glass tube using a pre-cooled glass Pasteur pipette. The fat layer on the surface was gently moved aside. Care was taken to avoid touching the pellet with the Pasteur pipette. The tube containing the supernatant was then vortexed and parafilmed. The tube was placed back in the ice bath.

Protein Determination

The Lowry method was used to determine the protein concentration of the cytosol.[312] Accurate measurement of the protein concentration of the cytosol is critical in calculating the binding capacity in fmol/mg of cytosol protein. Protein concentration of the cytosol was determined prior to the receptor assay so that the cytosol protein concentration could have been adjusted prior to the assay if necessary. The 0.1% crystalline BSA standard was supplied in the DSL estrogen receptor assay kit for use in the protein assay.

Lowry Protein Assay

1. Twenty glass tubes (16 x 125 mm) were labeled and arranged in duplicate as follows: reagent blank; BSA standard 10, 20, 30, 40, and 50 µl; cytosol tubes 10 and 20 µl; and MTG-buffer 10 and 20 µl.

2. Two hundred µl of cytosol was transferred to a labeled glass tube (12 x 75 mm). The cytosol was diluted 1:2 with distilled water.

3. In order to correct for interference from the buffer, a small quantity of MTG-buffer was diluted 1:2 with distilled water.

4. A pipette was used to deposit 10, 20, 30, 40, and 50 µl of 0.1% BSA standard into the duplicate labeled tubes. In a similar fashion 10 and 20 µl of diluted cytosol were deposited into the duplicate labeled tubes. Also, 10 and 20 µl of diluted MTG-buffer were pipetted into the duplicate labeled tubes. The reagent blank tubes were left empty.

5. To each tube 0.5 ml of distilled water was added.

6. The Lowry Reagent was freshly prepared as follows: just prior to its use, 1 part 2% sodium tartrate and 1 part 0.5% copper sulfate ($CuSO_4.5H_2O$) was added to 100 parts 2% sodium carbonate in 0.1 N NaOH.

7. Into each tube was added 2.5 ml of well mixed, freshly prepared Lowry Reagent. This was mixed and incubated for 10 minutes at room temperature.

8. Folin and Cicalteu's Reagent was diluted 1:2 in distilled water and 0.3 ml added to each tube. This was mixed and incubated for 30 minutes at room temperature.

9. The spectrophotometer was set to 610 nm and adjusted with the reagent blanks. All standards and samples were read.

10. From a standard curve the protein concentrations of cytosol and buffer were determined by direct interpolation. Protein concentrations of the cytosol samples were averaged. The true cytosol protein concentration was determined by subtracting the value obtained with the buffer from that of the cytosol protein.

Estrogen Receptor Assay Procedure

1. If the cytosol protein concentration was more than 5 mg/ml, as determined from the protein assay, it was diluted with chilled MTG-buffer and the adjusted cytosol was re-assayed to verify the final protein concentration.

2. Twelve x 75 mm glass tubes were marked and arranged according to the following protocol: for the positive control and tissue sample, two total binding tubes and two nonspecific

binding tubes labeled for each of the six tracer levels. Also, two total count tubes were labeled for each of the six tracer levels.

3. Ten µl of diethylstilbestrol (DES) was pipetted into each of the nonspecific binding tubes.

4. Fifty µl of each level of I^{125}-estradiol tracer (2.5, 1.25, 0.75, 0.38, 0.19 and 0.10) was pipetted into appropriately labeled total count, total binding and nonspecific binding tubes.

5. One hundred µl of adjusted cytosol was pipetted into each total binding and nonspecific binding tube.

6. All tubes were vortexed gently and incubated overnight (16 to 24 hours) at 0-4°C.

7. Three hundred µl of well-mixed separating reagent (activated dextran-coated charcoal) was added to each total binding and nonspecific binding tube. The separating reagent was not added to the total count tubes.

8. The total binding and nonspecific binding tubes were vortexed gently and incubated for 15 minutes at 0-4°C.

9. The total binding and nonspecific binding tubes were centrifuged at 1500 rpm for 10 minutes in a refrigerated centrifuge.

10. The supernatants from the centrifuged total binding and nonspecific binding tubes were then decanted into appropriately labeled tubes (12 x 75 mm glass tubes).

11. The total count tubes, total binding tubes and the nonspecific binding tubes were counted in a gamma counter for one minute each.

RESULTS

Tissue from the TMJ complexes of 9 female subjects, 16 to 30 years of age, were examined for the presence of ERs. The presence of classical ER in the tissue of the TMJ complex in the human would support a hypothesis that estrogen has a direct influence on the physiology of the TMJ. Furthermore, a sexual dimorphism of ER concentration in the TMJ complex, as previously reported in the baboon, may explain the sex differences in the prevalence of TMJ disorders.

The assay method most universally accepted for the determination of ER concentration is the biochemical assay based on the binding of radioactively labeled estradiol to receptor sites. This assay method was employed in this study. The quantity of tissue required for the assay requires that an invasive procedure on the TMJ be done. Therefore, tissue could only be obtained from diseased TMJs in patients selected for surgery based on clinical findings and the patients' symptoms. The subject composition of the study was influenced by the fact that no male patients were surgically treated for a TMJ disorder during the study period. Therefore, the study sample consisted entirely of females with diseased TMJs.

The assay used for the study relies on the presence of unoccupied receptor sites for binding of the labeled estradiol. The assay cannot reliably measure receptor binding capacity in

the presence of high concentrations of endogenous or exogenous estrogens.[256] Unfortunately, because of the age and sex of the subjects, three subjects were taking exogenous estrogens in form of oral contraceptives. There is no research indicating the degree to which the results of these assays would be altered by the quantity of estrogen supplied in today's low dose oral contraceptives.

In the 9 TMJ complexes assayed no ER binding was observed (Table 2). Receptor binding was observed in all of the positive controls. The ER binding capacity of the positive controls were 144 fmol, 180 fmol, 232 fmol, 75 fmol, 112 fmol, 103 fmol and 85 fmol per milligram of cytosol protein (Table 3). It is generally considered that ER values less than 3 fmol/mg cytosol protein are negative and ER values greater than 20 fmol/mg cytosol protein are positive.

Protein Concentration of the Cytosols

The protein concentration of the cytosols of the tissue samples and positive controls were determined initially, so that adjustments to the protein concentration could be made if required for assay accuracy. If the cytosol protein concentration was more than 5 mg/ml, the cytosol was to be diluted with more chilled MTG-buffer and re-assayed to determine the adjusted cytosol protein concentration. In all cases the initial protein concentration of the cytosol was less than 5 mg/ml and no further adjustments of protein

concentration were required. The protein concentration of the cytosols of the study subjects were 2.09 mg/ml, 2.06 mg/ml, 2.82 mg/ml, 3.91 mg/ml, 3.73 mg/ml, 2.09 mg/ml, 1.89 mg/ml, 2.05 mg/ml and 1.74 mg/ml (Table 2). The protein concentrations of the cytosols of the positive controls were 2.70 mg/ml, 2.57 mg/ml, 2.74 mg/ml, 3.42 mg/ml, 2.19 mg/ml, 1.76 mg/ml and 2.33 mg/ml (Table 3).

Determination of the Binding Capacity of the Cytosols

The total count tubes, total binding tubes and the nonspecific binding tubes were all counted in the gamma counter for one minute. The reported counts have all been corrected for instrument background. These results are reported in Tables 4-19 under "counts per minute" (CPM) for both the subject and positive control cytosols.

Determination of the Counter Efficiency

The gamma counter efficiency was determined after each run. The total count result for level 1 of the I^{125}-estradiol tracer was used to determine the counter efficiency. The counter efficiency was determined as follows:

i. Total count of level 1 tracer = CPM/0.05 ml (the tube contains 0.05 ml of 2.5 µCi/ml I^{125}-estradiol tracer).

ii. DPM/ml = A.C. (µCi/ml) x 2.2 x 10^6 DPM/ml. Where the A.C. is the activity concentration of the level 1 tracer corrected for decay.

iii. Counter efficiency = (CPM/ml)/(DPM/ml).

The counter efficiency for each assay of the subject or control cytosol is reported at the bottom of Tables 4-19. The counter efficiency for I^{125}-estradiol was approximately 75%. The activity concentration of level 1 tracer was found in the DSL estrogen receptor assay kit used for that run.

Conversion of "Counts Per Minute" (CPM) to "Decays Per Minute" (DPM)

The CPM were converted to DPM by initially averaging the CPM for each set of duplicates of all levels of tracer for the total count, total binding and the nonspecific binding tubes. The CPM was converted to DPM using the calculated counter efficiency as follows:

DPM = Averaged CPM divided by Counter Efficiency

The calculated DPM for each of the six levels of I^{125}-estradiol tracer for total count, total binding and nonspecific binding for both the subject cytosols and the positive control cytosols is reported in Tables 4-19.

Calculation of the Bound:Free (B/F) Ratio

The bound:free ratio was calculated using the DPM values for total count, total binding and nonspecific binding for each level of I^{125}-estradiol tracer for both the subjects and positive controls. The bound:free ratio was calculated as follows:

$$\frac{B}{F} = \frac{\text{TOTAL BOUND DPM - NONSPECIFIC BOUND DPM}}{\text{TOTAL COUNT DPM - (TOTAL BOUND DPM - NONSPECIFIC BOUND DPM)}}$$

The bound:free ratio results calculated for both subject and positive control cytosols for each I^{125}-estradiol level is reported in Tables 4-19.

Calculation of the Specific Bound Value in fmol/ml

The specific bound value was calculated in fmol/ml. The calculation of this value requires the determination of the corrected specific activity for the I^{125}-estradiol tracer. The corrected specific activity is determined using the specific activity from the specifications given with the "estrogen receptor assay kit". This specific activity is corrected using a decay factor which is related to the difference between the calibration date of the I^{125}-estradiol tracer and the date the assay is done. The corrected specific activity of I^{125}-estradiol for each subject and control is reported at the bottom of Tables 4-19.

The specific bound value was then calculated as follows:

$$\text{Specific Bound}_{\text{fmol/ml}} = \frac{\text{TOTAL BOUND DPM - NONSPECIFIC BOUND DPM}}{\text{CORRECTED S.A. (DPM/fmol) x 0.15 ml}}$$

The specific bound values for the subject and positive control cytosols for each level of I^{125}-estradiol tracer is reported in Tables 4-19.

Determination of the Binding Capacity

The binding capacity of the subject and positive control cytosols were determined using a Scatchard plot. Binding capacities less than 3 fmol/mg cytosol protein are negative for

ERs and binding capacities greater than 20 fmol/mg cytosol protein are positive for ERs. Binding capacities between 3 and 20 fmol/mg cytosol protein are indeterminant.

A Scatchard plot was done for each subject and positive control by plotting the B/F values (Y axis) against the specific bound values (X axis). A microcomputer using a simple linear regression program attempted to fit the best straight line to these points connecting both axes. All the Scatchard plots of subject cytosols demonstrated one of the following situations that indicated a lack of specific binding of estradiol to estrogen receptors: a large degree of scatter and lack of linearity, a positive slope of the line (slope should always be negative) or specific bound levels of 0 fmol/ml. The binding capacities of all the subject cytosols were therefore 0 fmol/mg of cytosol protein (Table 2). Scatchard plots were fitted to all positive control cytosols and the binding capacities were calculated as follows:

$$\text{Binding Capacity (fmol/mg)} = \frac{\text{X INTERCEPT (fmol/ml) x REACTION VOLUME (ml)}}{\text{CYTOSOL VOL (ml) x PROTEIN CONCENTRATION (mg/ml)}}$$

Since $\dfrac{1}{\dfrac{\text{Cytosol Volume (ml)}}{\text{Reaction Volume (ml)}}}$ **equals** $\dfrac{1}{\dfrac{0.10}{0.15}}$

For this assay, the equation can be simplified to:

$$\text{Binding Capacity (fmol/mg)} = \frac{\text{X INTERCEPT (fmol/ml) x 1.50}}{\text{ADJUSTED PROTEIN CONCENTRATION}}$$

The X-intercept values extrapolated from Scatchard plots of the positive controls are reported in Table 3. The positive control Scatchard plots are Figures 1-7.

The binding capacity of the positive controls were calculated to be 144 fmol, 180 fmol, 232 fmol, 75 fmol, 112 fmol, 103 fmol and 85 fmol per milligram of cytosol protein (Table 3). These values are all consistent with the presence of classical estrogen receptors.

Table 1: Study Group

No.	Subject/ Unit No.	Sex/ Age	Diagnosis	Date of Operation	Date of Assay	Exogenous Estrogens
1	MA 881506	F 18	L. TMJ anterior disc displacement with reduction	88-05-12	88-05-17	NO
2	MB 885571	F 27	R. TMJ anterior disc displacement without reduction	88-10-05	88-10-13	NO
3	AV 873473	F 22	R. TMJ anterior disc displacement without reduction	88-12-01	88-12-08	BCP-Triphasil
4	JW 892869	F 16	R. TMJ anterior disc displacement without reduction	89-01-04	89-01-06	NO
5	JC 895708	F 26	L. TMJ anterior disc displacement without reduction	89-01-05	89-01-06	NO
6	KB 896115	F 24	L. TMJ anterior disc displacement without reduction	89-01-26	89-01-31	BCP-Triphasil
7	MT 896092	F 17	L. TMJ anterior disc displacement without reduction	89-02-08	89-03-09	NO
8	CG 839198	F 21	L. TMJ anterior disc displacement without reduction	89-02-15	89-03-09	BCP-Ortho777
9	LB 844706	F 30	L. TMJ anterior disc displacement without reduction	89-03-22	89-04-07	NO

Table 2: Estrogen Receptor Binding Capacity of Tissue from TMJ Complexes

No.	Subject	TMJ Tissue Submitted	Date Assayed	Tissue Weight (mg)	MTG-Buffer Volume (ml)	Protein Concen-ration of Cytosol (mg/ml)	Estrogen Receptor Binding Capacity (fmol/mg)
1	MA	left	88-05-17	483	4	2.09	0
2	MB	right	88-10-13	203	4	2.06	0
3	AN	right	88-12-08	475	4	2.82	0
4	JW	right	89-01-06	255	4	3.91	0
5	JC	left	89-01-06	363	4	3.73	0
6	KB	left	89-01-31	247	4	2.09	0
7	MT	left	89-03-09	65	4	1.89	0
8	CG	left	89-03-09	226	4	2.05	0
9	LB	left	89-04-07	125	4	1.74	0

Table 3: Estrogen Receptor Binding Capacity of Positive Controls

Positive Control Number	Subject	Date of Assay	Tissue Weight (mg)	Total Cytosol Volume (ml)	Protein Concentration Cytosol (mg/ml)	X Intercept Value from Scatchard Plot (fmol/mg)	Estrogen Receptor Binding Capacity (fmol/mg)
1	MA	88-05-17	500	3	2.70	260	144
2	MB	88-10-13	500	3	2.57	309	180
3	AV	88-12-08	500	3	2.74	425	232
4	JC/JW	89-01-06	500	3	3.42	171	75
5	KB	89-01-31	500	3	2.19	163	112
6	MT/CG	89-03-09	500	3	1.76	121	103
7	LB	89-04-07	500	3	2.33	132	85

NOTE: Positive controls 4 and 6 are controls for two subjects each. Subjects JC and JW were both done in the same run (#4) as were subjects MT and CG (#6).

Table 4: Subject MA

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	128175 123913	167834	10027.3	10027.3	10323 9611	13286	0	0	0
Level 2	70641 64739	90133	5909.0 5474.0	7586.7	5883.0 6046.0	7950.6	0	0	0
Level 3	35156 34123	46123	3190.4 2817.1	4004.0	3205.5 2921.3	4083.5	0	0	0
Level 4	18073 18513	24358	1569.9 1593.0	2108.1	1464.0 1863.0	2217.4	0	0	0
Level 5	10051 9049.0	12716	801.0 737.0	1025.0	1092.5 801.1	1262.0	0	0	0
Level 6	6055.0 5886.1	7960	501.7 560.4	707.9	415.7 435.0	567.0	140.9	1.527	.01802

Corrected Specific Activity of I^{125} Estradiol = 603 DPM/fmol

Cytosol Protein Concentration = 2.09 mg/ml

Counter Efficiency = 75.10%

Table 5: Positive Control (MA)

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	128175 123913	167834	23143 23955	31390	10780 10285	14039	17351	191.69	.1152
Level 2	70641 64739	90133	20080 19694	26509	5978.2 5807.2	7854.9	18654	206.09	.2606
Level 3	35156 34123	46123	16217 15160	20913	2720.0 2838.0	3704.1	17208	190.11	.5941
Level 4	18073 18513	24358	10029 9789.0	13208	1540.0 1617.4	2104.8	11103	122.68	.8362
Level 5	10051 9049.0	12716	5215.5 5577.6	7193.0	778.9 774.0	1034.9	6158.1	68.04	.9371
Level 6	6055.0 5886.1	7960	2789.0 2439.0	3484.4	464.0 415.0	585.8	2898.6	32.02	.5729

Corrected Specific Activity of I^{125} Estradiol = 603 DPM/fmol

Cytosol Protein Concentration = 2.70 mg/ml

Counter Efficiency = 75.10%

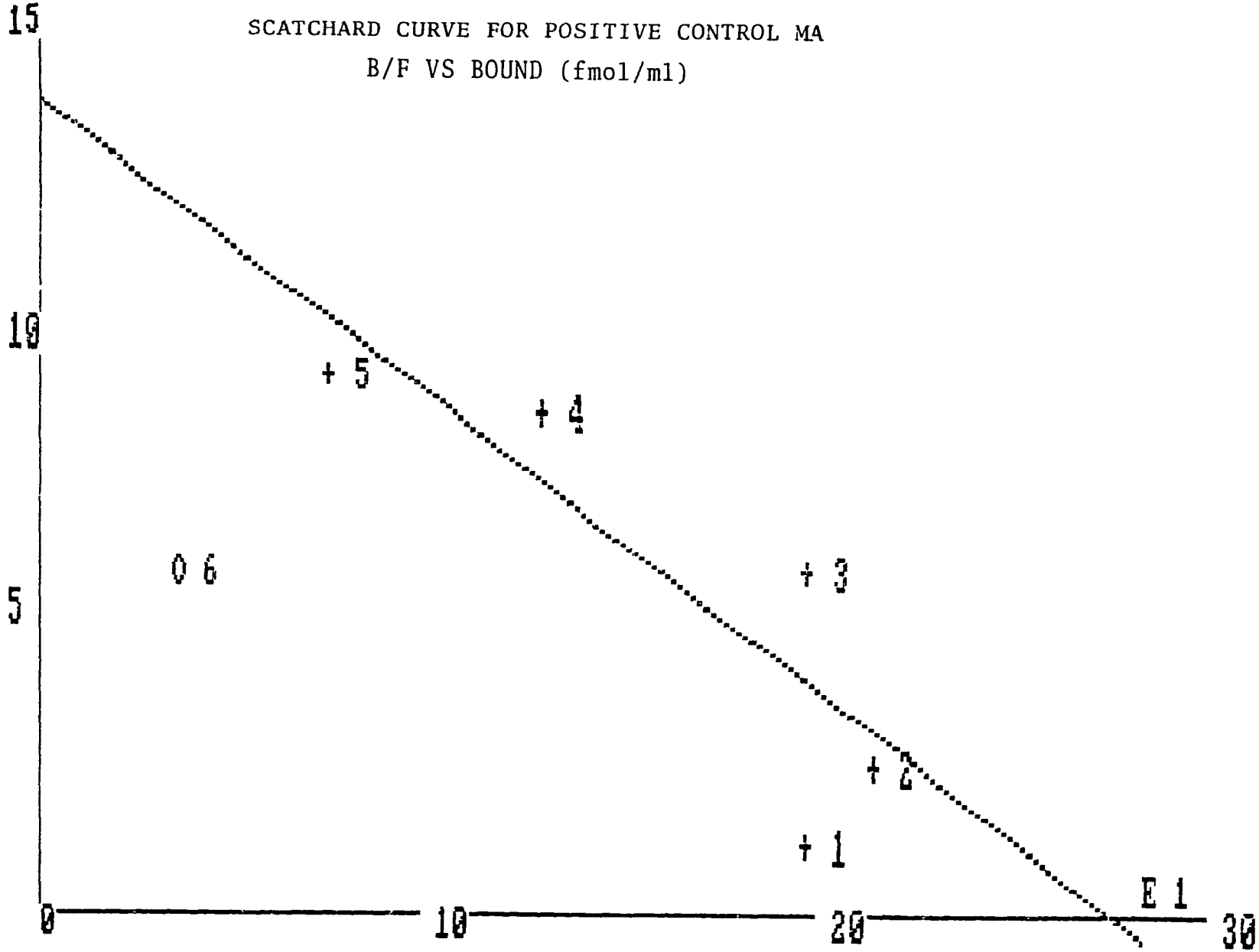
SCATCHARD CURVE FOR POSITIVE CONTROL MA
B/F VS BOUND (fmol/ml)
15
10
5
0
10
20
30
+ 5
+ 4
0 6
+ 3
+ 2
+ 1
E 1

FIGURE 1

Table 6: Subject MB

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	103018 96267	132824	4815.9 3716.0	5686.5	4621.0 5570.0	6792.3	0	0	0
Level 2	52903 56501	72918	2702.0 2827.0	3685.1	3105.4 2771.6	3867.0	0	0	0
Level 3	22947 23988	31283	1200.7 1340.3	1693.6	1272.5 1329.0	1733.9	0	0	0
Level 4	14335 14108	18957	724.0 670.0	929.1	654.0 624.0	851.5	851.5	77.6	.004110
Level 5	7131 7096	5101.6	389.4 315.6	469.9	371.2 181.1	368.2	101.7	2.291	.02034

Corrected Specific Activity of I^{125} Estradiol = 296 DPM/fmol

Cytosol Protein Concentration = 2.06 mg/ml

Counter Efficiency = 75.018%

Table 7: Positive Control (MB)

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	103018 96267	132824	15323 14496	19875	7069.0 5948.0	8675.9	11199	251.95	.0921
Level 2	52903 56501	72918	12993 12686	17115	3642.5 3505.0	4763.8	12351	277.89	.2039
Level 3	22947 23988	31283	10223 10142	13574	1304.9 1672.0	1984.1	11590	260.75	.5886
Level 4	14335 14108	18957	8039 8298	10889	757.0 836.0	1061.8	9827.2	221.09	1.0764
Level 5	7131 7096	9482.3	4614.4 4565.0	6518.3	395.5 402.6	531.95	5986.4	125.69	1.4343
Level 6	3894.4 3759.9	5101.6	2512.6 2425.0	3290.9	215.0 206.0	280.60	3010.4	67.75	1.4401

Corrected Specific Activity of I^{125} Estradiol = 296 DPM/fmol

Cytosol Protein Concentration = 2.57 mg/ml

Counter Efficiency = 75.018%

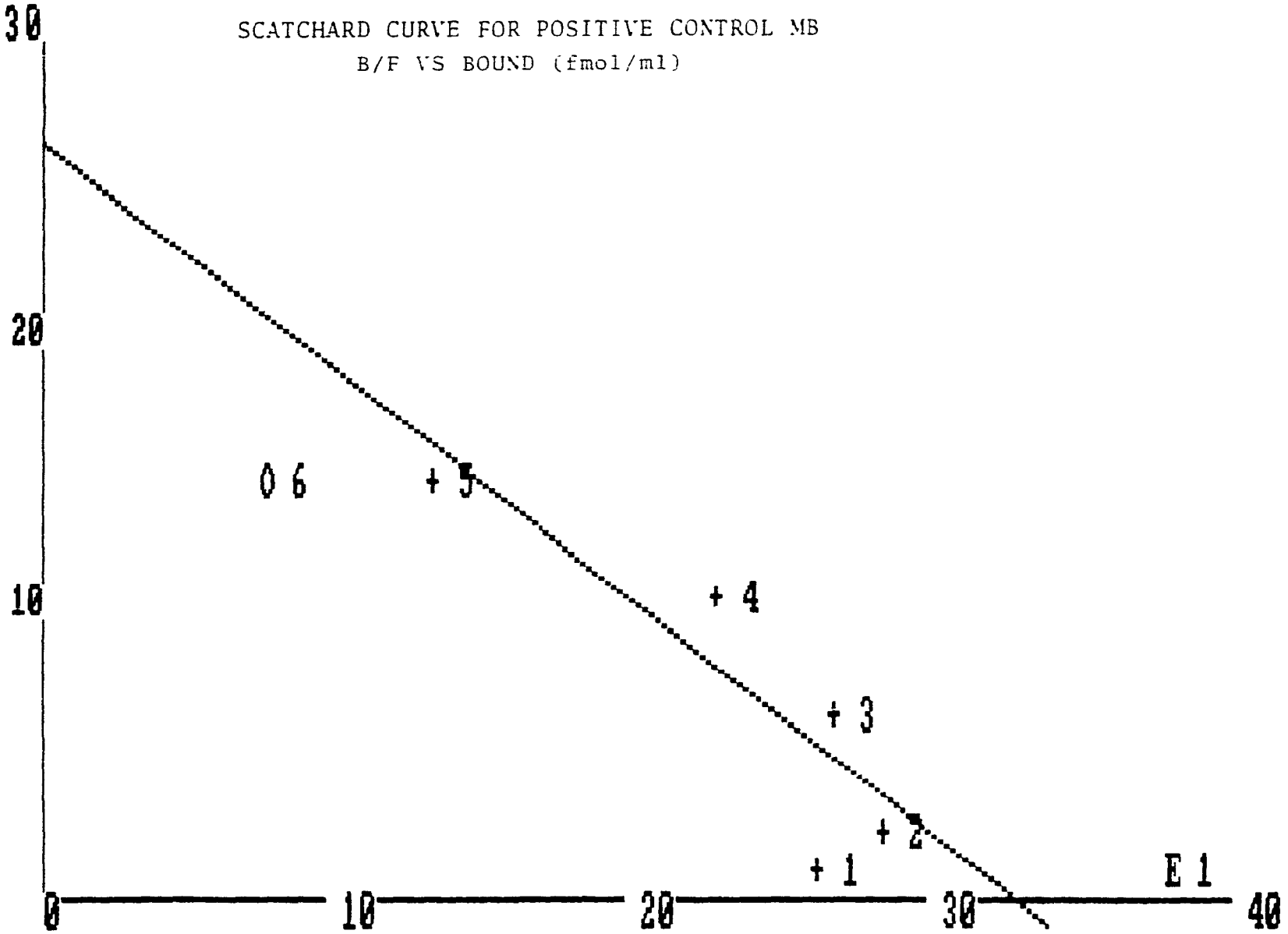
SCATCHARD CURVE FOR POSITIVE CONTROL MB
B/F VS BOUND (fmol/ml)
30
20
10
0
10
20
30
40
0 6
+ 5
+ 4
+ 3
+ 2
+ 1
E 1

FIGURE 2

Table 8: Subject AV

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	103796 96654	133600	8715.4 7697.8	10939	13324.9 8159.0	14319	0	0	0
Level 2	53259 49768	68668	5458.0 5771.0	7484.1	4620.0 4385.0	6001.9	1482.2	31.15	.02206
Level 3	26174 25732	34596	1775.2 2340.7	2743.3	2322.5 2251.7	3048.7	0	0	0
Level 4	13063 13449	17670	677.7 1051.0	1152.2	1341.0 977.0	1545.0	0	0	0
Level 5	6988 6715	9133.1	639.0 647.0	857.2	682.8 494.6	748.8	108.4	2.287	.01201
Level 6	3666.8 3714.3	4919.5	422.8 236.7	439.5	312.6 363.0	450.3	0	0	0

Corrected Specific Activity of I^{125} Estradiol = 316 DPM/fmol

Cytosol Protein Concentration = 2.82 mg/ml

Counter Efficiency = 75.0%

Table 9: Positive Control (AV)

I^{125} Estradiol	Total Count		Total Binding		Non Specific Binding		Specific Binding		
	CPM	mean DPM	CPM	mean DPM	CPM	mean DPM	mean DPM	fmol/ml	B/F
Level 1	103796 96654	133600	18168 17916	24050	7376.1	9832.3	14218	300.31	.1191
Level 2	53259 49768	68668	13545.4 15041.5	19053	3910.6 3924.0	5221.8	13931	252.15	.2522
Level 3	26174 25732	34596	11245 11778	15345	1719.0	2291.4	13053	275.72	.6060
Level 4	13063 13449	17670	7533.9 7098.9	9752.8	956.9 904.3	1240.5	8512	179.79	.9294
Level 5	69880 6715	9133.1	3964.2 3829.0	5194.2	523.0 488.0	673.9	4420	95.48	.9801
Level 6	3666.8 3714.3	4919.5	1975.0 1818.0	2528.1	265.0 186.1	300.7	2227	47.05	.8276

Corrected Specific Activity of I^{125} Estradiol = 316 DPM/fmol

Cytosol Protein Concentration = 2.74 mg/ml

Counter Efficiency = 75.0%

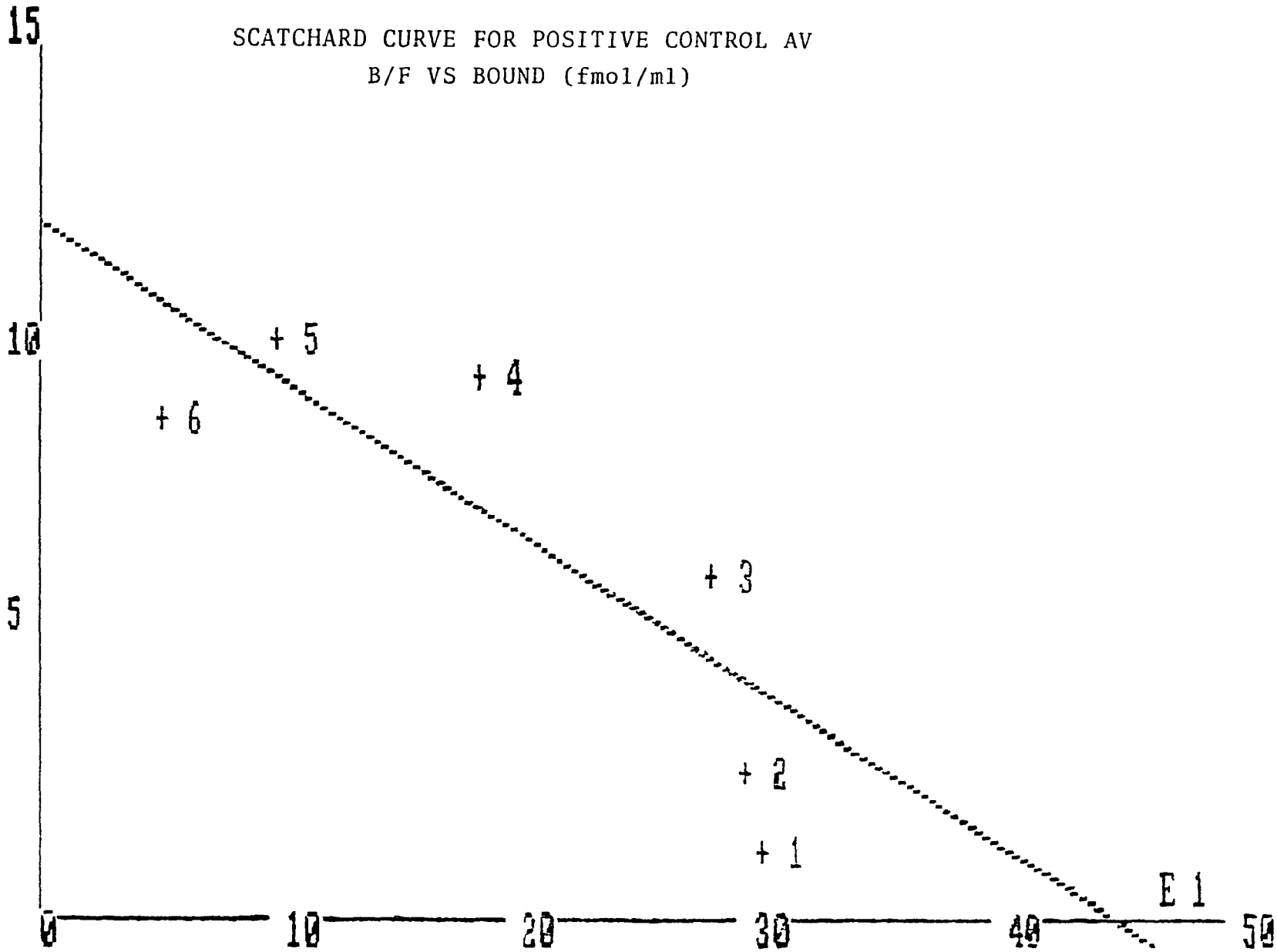
SCATCHARD CURVE FOR POSITIVE CONTROL AV
B/F VS BOUND (fmol/ml)
15
10
5
0
10
20
30
40
50
+ 1
+ 2
+ 3
+ 4
+ 5
+ 6
E 1

FIGURE 3

Table 10: Subject JW

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	155409 157284	208410	4453 3063	5009	4388 4771	5891	0	0	0
Level 2	73780 70657	96267	1847 1598	2296	1740 2100	2559	0	0	0
Level 3	39928 38459	52245	1095 1127	1481	873.0 906.3	1186	295	3.469	.005679
Level 4	18507 17211	23806	483.5 479.0	641.5	481.0 565.0	697.2	0	0	0
Level 5	9811.0 9783.0	13059	243.0 203.0	297.3	217.5 185.1	201.3	96.0	1.129	.007406
Level 6	5452.2 5148.7	7065.5	155.8 162.9	212.4	137.6 128.0	177.0	35.4	.4162	.005035

Corrected Specific Activity of I^{125} Estradiol = 567 DPM/fmol

Cytosol Protein Concentration = 3.91 mg/ml

Counter Efficiency = 75.019%

Table 11: Subject JC

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	155409 157284	208410	3230 4844	5381	3589 4163	5167	214	2.516	.001028
Level 2	73780 70657	96267	2307 1553	2573	1764 1501	2176	397	4.668	.004141
Level 3	39928 38459	52245	920.5 837.0	1171	876.0 1052.0	1285	0	0	0
Level 4	18507 17211	23806	372.0 473.0	563.2	443.1 503.7	631.1	0	0	0
Level 5	9811.0 9783.0	13059	349.0 217.5	377.5	192.2 188.0	253.4	124.1	1.459	.009594
Level 6	5452.2 5148.7	7065.5	135.0 91.0	150.7	125.0 127.0	167.9	0	0	0

Corrected Specific Activity of I^{125} Estradiol = 567 DPM/fmol

Cytosol Protein Concentration = 3.73 mg/ml

Counter Efficiency = 75.019%

Table 12: Positive Control (JW) (JC)

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	155409 157284	208410	12344	16429	4348	5797	10632	124.97	.0538
Level 2	73780 70657	96267	11022 11955	14648	3762	5015	9633	121.06	.1198
Level 3	39928 38459	52245	9835 10139	13313	1266 1418	1789.2	11524	135.46	.2830
Level 4	18507 17211	23806	7648	10195	622 625	831.2	9363.8	110.07	.6483
Level 5	9811.0 9783.0	13059	5679	7570.0	323 302	416.6	7153.5	84.09	1.2113
Level 6	5452.2 5148.7	7065.5	3190 3109	4198.3	269 227	330.6	3867.7	45.46	1.2097

Corrected Specific Activity of I^{125} Estradiol = 567 DPM/fmol

Cytosol Protein Concentration = 3.42 mg/ml

Counter Efficiency = 75.019%

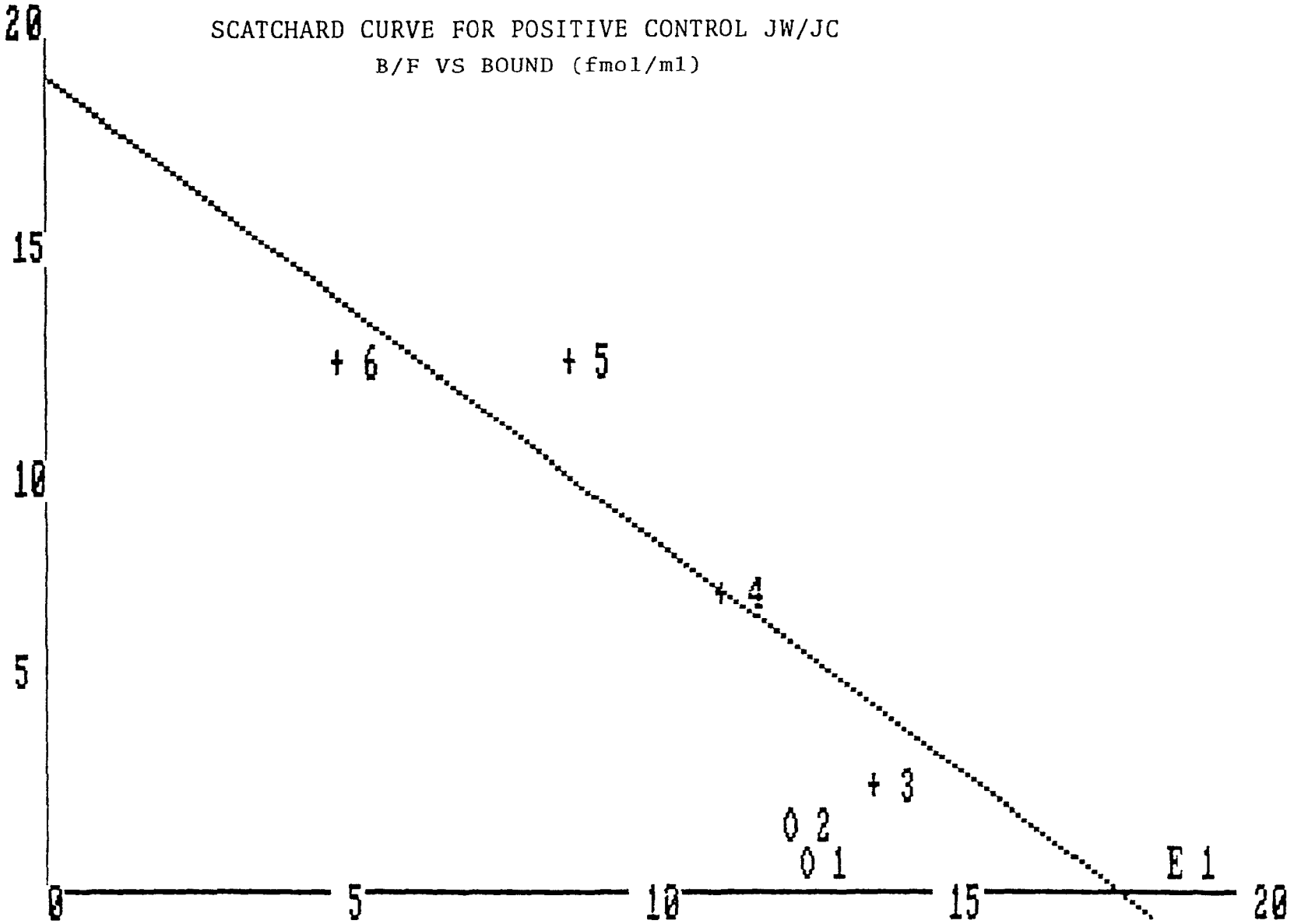
SCATCHARD CURVE FOR POSITIVE CONTROL JW/JC
B/F VS BOUND (fmol/ml)
20
15
10
5
0
5
10
15
20
+ 6
+ 5
+ 4
+ 3
0 2
0 1
E 1

FIGURE 4

Table 13: Subject KB

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	97740 103245	133957	3723 3962	5122	4322 3531	5234	0	0	0
Level 2	51121 52724	69213	1641 2155	2530	2001 2231	2821	0	0	0
Level 3	27860 27693	37027	1324 994.3	1545	869.9 1330	1466	79	1.313	.002138
Level 4	13604 13454	18034	612.0 465.0	717.8	362.0 414.0	517.2	200.6	3.335	.01125
Level 5	7135 6769	9267	352.0 202.3	369.5	290.3 252.9	362.1	7.4	0.1230	.0007991
Level 6	3583 3755	4892	125.4 137.0	174.9	167.0 171.0	225.3	0	0	0

Corrected Specific Activity of I^{125} Estradiol = 401 DPM/fmol

Cytosol Protein Concetration = 2.09 mg/ml

Counter Efficiency = 75.019%

Table 14: Positive Control (KB)

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	97740 103245	133957	8740 9837	12382	3982 4354	5557	6825	113.50	.0537
Level 2	51121 52724	69213	8215 8799	11341	2065 2033	2732	8609	143.15	.1420
Level 3	27860 27693	37027	7251 7293	9694	1058 1050	1405	8289	137.83	.2884
Level 4	13604 13454	18034	6671 6318	8657	414 543	637.8	8019	133.35	.8007
Level 5	7135 6769	9267	4283 4163	5629	310 286	397.2	5232	87.0	1.2967
Level 6	3583 3755	4892	2137 2367	3002	146 138	189.3	2813	46.77	1.3569

Corrected Specific Activity of I^{125} Estradiol = 401 DPM/fmol

Cytosol Protein Concentration = 2.19 mg/ml

Counter Efficiency = 75.019%

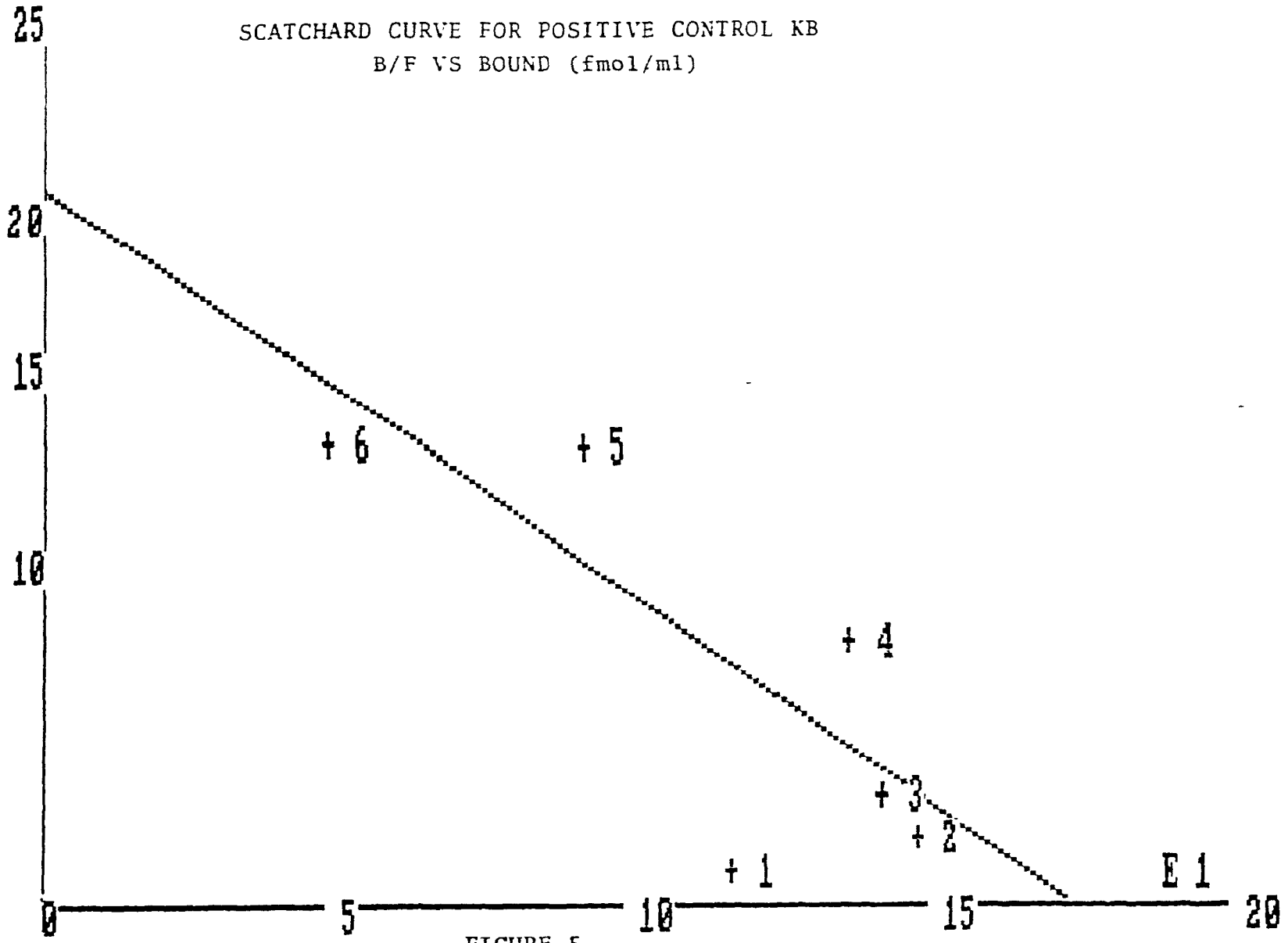
SCATCHARD CURVE FOR POSITIVE CONTROL KB
B/F VS BOUND (fmol/ml)
25
20
15
10
0
5
10
15
20
+ 6
+ 5
+ 4
+ 3
+ 2
+ 1
E 1

FIGURE 5

Table 15: Subject MT

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	138537 134363	181888	4757 5918	7115	5142 4842	6654	361	6.723	.001989
Level 2	66221 65151	87561	2880 2670	3699	5835 2590	4213	0	0	0
Level 3	33513 32061	43705	1522 1236	1838	1401 1313	1809	28	.5400	.0006639
Level 4	16534 16643	22112	603.0 622.0	816.5	699.0 641.3	893.3	0	0	0
Level 5	9597 8655	12165	401.6 353.0	502.9	280.2 308.0	392.1	110.8	2.063	.009191
Level 6	4780 5338	6745	132.0 185.0	211.3	145.0 184.0	219.3	0	0	0

Corrected Specific Activity of I^{125} Estradiol = 358 DPM/fmol

Cytosol Protein Concentration = 1.89 mg/ml

Counter Efficiency = 75.019%

Table 16: Subject CG

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	138537 134363	181888	8199 8511	11137	6675 7406	9385	1752	32.62	.009736
Level 2	66221 65151	87561	2859 5360	5478	3849 4179	5351	127	2.365	.001453
Level 3	33513 32061	43705	2114 1519	2422	1649 1451	2066	356	6.629	.008212
Level 4	16534 16643	22112	1166 820	1324	840.0 578.0	945.1	379	7.058	.017439
Level 5	9597 8655	12165	720.0 585.0	869.8	397.5 439.0	557.6	312	5.810	.02632
Level 6	4780 5338	6745	265.0 393.5	438.9	188.1 242.0	286.7	152	2.831	.02305

Corrected Specific Activity of I^{125} Estradiol = 358 DPM/fmol

Cytosol Protein Concentration = 2.05 mg/ml

Counter Efficiency = 75.019%

Table 17: Positive Control (MT) (CG)

I^{125} Estradiol	Total Count CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	138537 134363	181888	11115 11812	15282	6579 6763	8893	6389	119.01	.0364
Level 2	66221 65151	87561	7834 7987	10545	3148 3147	4196	6349	118.28	.0728
Level 3	33513 32061	43705	6475 6761	8822	1908 2821	3153	5669	105.63	.1491
Level 4	16534 16643	22112	5603 5662	7509	1134 884	1345	6164	114.83	.3865
Level 5	9597 8655	12165	4672 4783	6302	762 419	787.1	5515	102.73	.8292
Level 6	4780 5338	6745	2900 2094	3329	194 221	276.6	3052	56.86	.8267

Corrected Specific Activity of I^{125} Estradiol = 358 DPM/fmol

Cytosol Protein Concentration = 1.76 mg/ml

Counter Efficiency = 75.019%

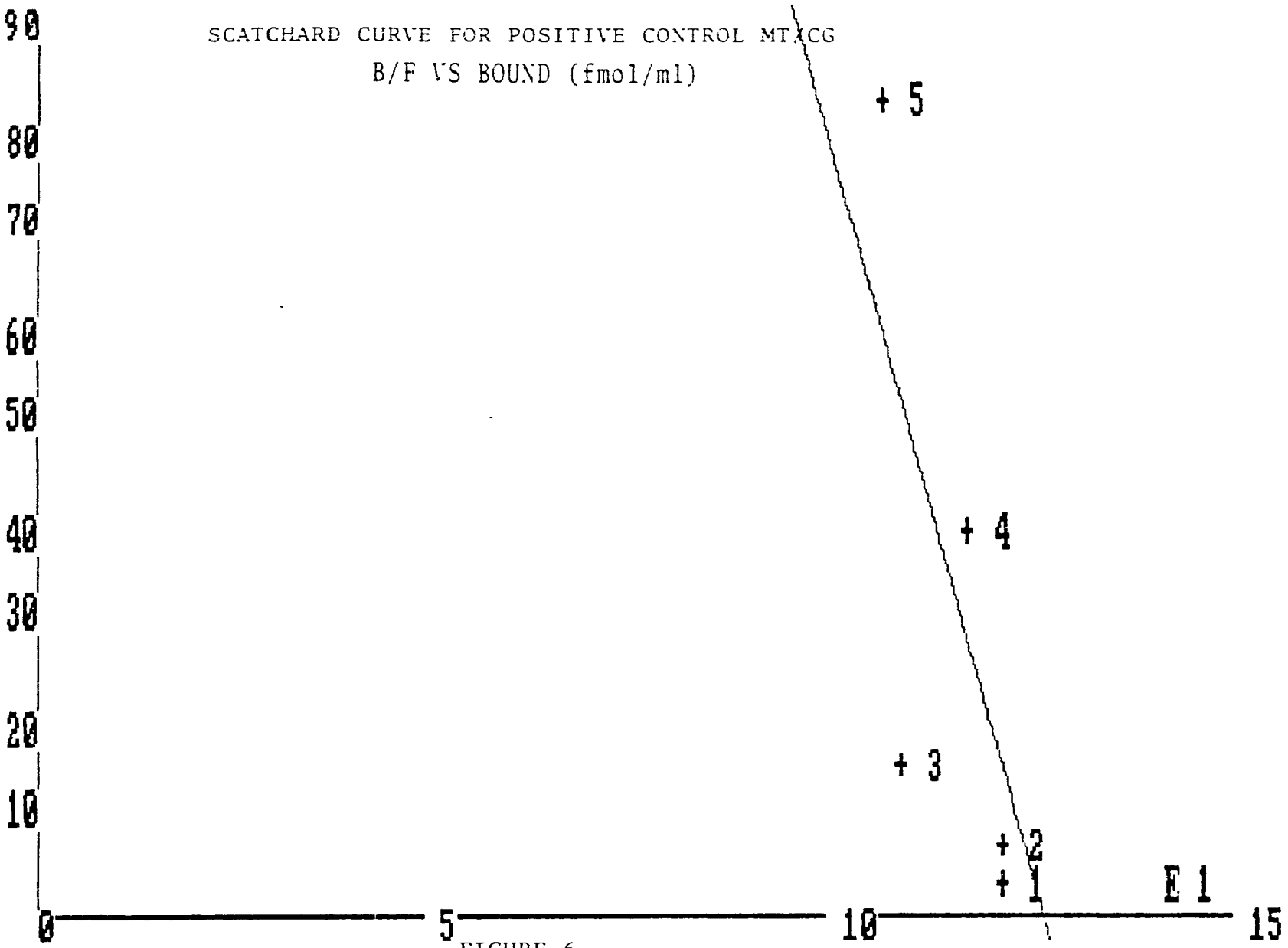
SCATCHARD CURVE FOR POSITIVE CONTROL MTCG
B/F VS BOUND (fmol/ml)
90
80
70
60
50
40
30
20
10
0
5
10
15
+ 5
+ 4
+ 3
+ 2
+ 1
E 1

FIGURE 6

Table 18: Subject LB

I^{125} Estradiol	Total Count mean CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	102666	136853	6663 6958	9079	5817 5361	7450	1629	40.98	.01205
Level 2	51457	68592	2713 2821	3688	2519 2715	3488	200	5.031	.002924
Level 3	24545	32718	1174 1206	1586	1143 1141	1522	64	1.610	.001960
Level 4	12286	16377	670.6 549.0	790.4	581.0 574.0	769.8	20.6	.5182	.001259
Level 5	6346	8459	363.0 303.0	443.9	268.1 279.2	364.8	79.1	1.990	.009392
Level 6	3947	5261	174.0 174.0	231.9	167.9 183.0	233.9	0	0	0

Corrected Specific Activity of I^{125} Estradiol = 265 DPM/fmol

Cytosol Protein Concentration = 1.74 mg/ml

Counter Efficiency = 75.019%

Table 19: Positive Control LB

I^{125} Estradiol	Total Count mean CPM	Total Count mean DPM	Total Binding CPM	Total Binding mean DPM	Non Specific Binding CPM	Non Specific Binding mean DPM	Specific Binding mean DPM	Specific Binding fmol/ml	Specific Binding B/F
Level 1	102666	136853	8047 8146	10793	5279 5048	6883	3910	98.53	.0294
Level 2	51457	68592	6133 5721	7901	2496 2748	3496	4405	111.03	.0686
Level 3	24545	32718	4922 4826	6497	1250 1344	1729	4768	120.17	.1706
Level 4	12286	16377	4175 4214	5591	633 702	910.2	4681	117.98	.4003
Level 5	6346	8459	3507 3315	4547	339 388	485.2	4062	102.38	.9239
Level 6	3947	5261	2307 2214	3013	185 152	224.6	2788	70.28	1.1278

Corrected Specific Activity of I^{125} Estradiol = 265 DPM/fmol

Cytosol Protein Concentration = 2.33 mg/ml

Counter Efficiency = 75.019%

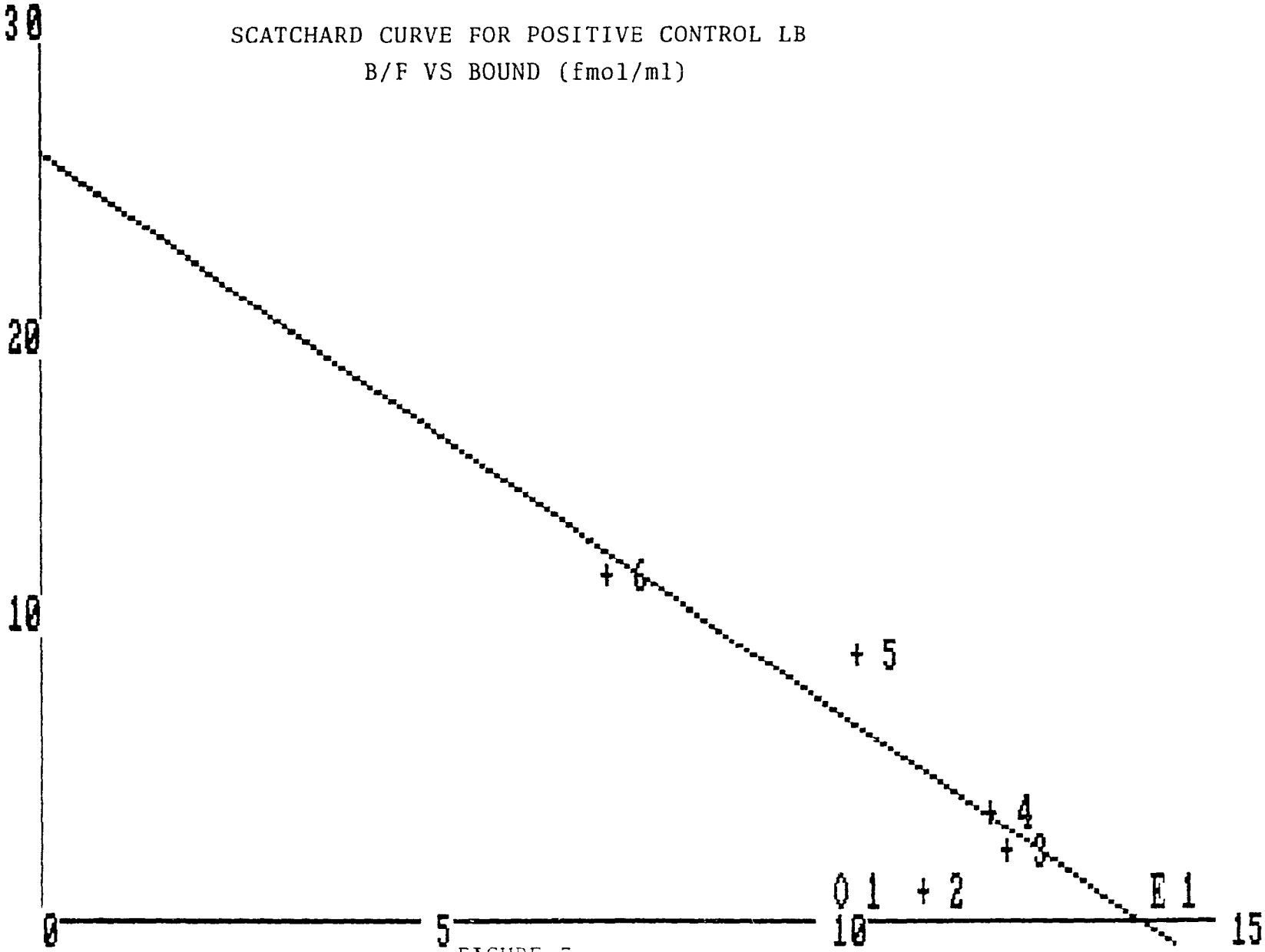
SCATCHARD CURVE FOR POSITIVE CONTROL LB
B/F VS BOUND (fmol/ml)
30
20
10
0
5
10
15
+ 6
+ 5
+ 4
+ 3
0 1 + 2
E 1

FIGURE 7

DISCUSSION

There is general agreement that the overwhelming majority of patients seeking treatment for TMJ disorders are female.[1-33] Furthermore, population studies indicate that women show more signs and complain of more symptoms of TMJ disorders than men.[1,3,15,100,102,105,117,126,130,139,140-163,166] These findings led Butler, Folke and Bandt (1975)[19] to suggest that TMJ syndrome may be a sex linked condition. Subsequently, a sexual dimorphism of estrogen receptors in the TMJ complex of the baboon was demonstrated.[39,40] A pathophysiological basis for observed sex differences of TMJ disorders was proposed on the basis of direct estrogenic effects on the tissue of the TMJ complex. A target tissue must have ERs in order to be directly influenced by estrogen.[51,54-49] If the tissue of the human TMJ complex is responsive to the direct effects of estrogen, it should be possible to demonstrate the presence of ERs in this tissue.

This study demonstrated the absence of ER binding in the TMJ complexes of the subjects studied. Therefore, there is no evidence from this study or in the literature to support a direct influence of estrogen on the TMJ complex of the human.

The subjects included in this study were exclusively females. This was because no male patients were treated surgically during the study period. It was therefore not

possible to demonstrate sex differences in receptor binding between human males and females. It is interesting to note that the sexual dimorphism of ER distribution, previously demonstrated in the baboon, consisted of a complete absence of receptor binding in the male and an abundance of receptor binding in the female TMJ complex.[39,40] All female baboons showed ER binding. If these findings can be extrapolated to humans, then it should have been possible to demonstrate receptor binding in at least some of the female subjects in the present study.

The TMJ complexes studied were diseased in all cases. This was necessitated by the quantity of tissue required for the assay. One could argue that the risk factor for acquiring TMJ disease is the absence and not the presence of ERs in the TMJ complex. In order to correctly assess whether the presence of ERs in the TMJ complex is a risk factor for TMJ disease, an assay which could be performed on normal TMJs would have to be used. In the future, the recently described enzyme-immunochemical assay could possibly be used to test for receptor binding in both normal male and female human TMJs using a needle biopsy technique.[91,92,256,289,291] If the morbidity from a needle biopsy proved to be too great to use on normal TMJs, biochemical studies could be done on freshly harvested tissue from organ donors. Since ER assays based on monoclonal antibody to receptor protein can be done on lightly

fixed tissue, it is possible that TMJ tissue from fresh cadavers could be assayed for ERs using an immunological technique.

The assay used for this study relies on unoccupied receptors to bind to radioactively labeled estradiol. This assay cannot, therefore, reliably measure receptor binding capacity in the presence of high concentrations of endogenous or exogenous (oral contraceptives) estrogens.[256] Recently described assays based on monoclonal antibodies to ER protein could be used to alleviate this problem in future studies concerning ER binding.[253,256,293]

The results of this study differ from those of Aufdermorte et al,[39] in that they reported the presence of ER binding in all of their female baboon subjects. The method they used to assay for ER was an autoradiographic technique using ^{3}H-estradiol-17β. It has been suggested that these morphological techniques may not measure the classical Type I estrogen binding sites that are measured by biochemical determinations.[59] The significance of receptor binding demonstrated by morphological techniques has not yet been determined. Often there is an apparent lack of agreement between morphological and biochemical techniques.[274,284,286] The ER binding observed in the TMJ of the female baboon may not be classical Type 1 binding, that results in a demonstrable biological effect, and is therefore of unknown significance.

The absence of ERs in a tissue does not rule out an indirect influence of the hormone on that tissue. Estrogen may act through a different tissue or organ system to indirectly effect a response. ERs have not been identified in bone.[313] In vitro studies have demonstrated that estrogens have no direct effect on bone metabolism.[314,315] Yet clinical studies have repeatedly demonstrated that estrogens are extremely important for the preservation of mineralized bone.[201-210] Therefore, a risk factor for acquiring a TMJ disorder may be an elevated serum ovarian hormone level, that acts through an indirect mechanism effecting the maintenance, repair and/or pathogenesis of the TMJ. For example, there is a clinical correlation between the pathological states of the periodontium and circulating levels of sex steroid hormones.[72, 190] A study to determine if elevated serum levels of sex steroid hormones are a risk factor for the development of TMJ disorders would be of clinical significance. A positive result could imply an indirect effect on the TMJ complex.

What would be the clinical implications of discovering that altered levels of serum ovarian hormone and/or the presence of ER in the tissue of the TMJ complex of the human are risk factors for developing a TMJ disorder? A positive finding would certainly influence recommendations concerning the administration of exogenous estrogen and progesterone (for example, oral contraceptives) in patients with clinical signs and symptoms of TMJ disorders. The use of anti-estrogen

therapy in certain selected cases of TMJ disorders could be indicated, as well as replacement therapy in other selected cases depending upon the identified risk factor and the nature of the disease process.

This study demonstrated the absence of ER binding in the diseased TMJ complex of the human female. This finding is in contrast to an animal study in which ERs were demonstrated in the TMJ complex of the female baboon using an autoradiographic morphological method.[39] The results of this human study do not support the hypothesis of a direct effect of estrogen on human TMJ tissue. Further studies are indicated to investigate a direct or indirect mechanism for the influence of ovarian hormones on the maintenance, repair and/or pathogenesis of the TMJ in humans.

SUMMARY AND CONCLUSION

This study was undertaken to determine whether the human TMJ complex contains classical ERs. If the human TMJ complex contains ERs, it would be reasonable to conclude that the TMJ complex may be responsive to the direct effects of estrogens.

The method used involved the harvesting of TMJ tissue consisting of capsule, disc, retrodiscal tissue and fibrocartilage from the TMJs of 9 female subjects, 16 to 30 years of age, undergoing open TMJ arthrotomy for treatment of their internal derangements. Biochemical ER assays were performed on the tissue using 17β-I^{125}-estradiol. Specific binding was assessed from the evaluation of total and nonspecific tissue binding of estradiol. The tissue estradiol binding capacity was calculated using multiple point Scatchard plot analysis on the tissue samples and the positive controls (rabbit uterus).

The results demonstrated the presence of ERs in the positive controls and the absence of ERs in the tissue from the TMJ complexes of the subjects.

It was concluded that there is no evidence to support a direct influence of estrogen on the tissue of the TMJ complex in humans. However, further investigations utilizing either biochemical assays on fresh tissue from organ donors or

monoclonal antibody assays to ER protein on tissue from fresh cadavers could provide information on botn normal and abnormal male and female TMJs. Also, an investigation designed to determine if abnormal serum ovarian hormone concentration is a risk factor for TMJ disorders could provide evidence for an indirect hormonal influence on the human TMJ complex.

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