EXTREMELY SKEWED X-CHROMOSOME INACTIVATION IN JUVENILE IDIOPATHIC ARTHRITIS

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ABSTRACT

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Juvenile idiopathic arthritis (JIA) is the most common childhood rheumatic disease with female predominance and an incidence between 7-21/100,000. There are several explanations for the reason of disease development, such as environmental factors that trigger autoimmunity and genetic basis. The genetic basis of JIA is not well defined. It rarely manifests familial recurrence. But the monozygotic twin data suggest that there is a considerable genetic basis, which is likely to involve multiple epigenetic events. It was proposed that a disturbance in mosaicism of females may cause autoimmune disease development. Recently, in our lab, an association between extremely skewed X-chromosome inactivation (XCI) patterns and female predisposition to autoimmunity was identified. Since JIA is thought to have an autoimmune etiology, we hypothesized that skewed XCI might play a role in the disease development. To determine XCI status, androgen receptor locus was analyzed by methylation sensitive *Hpa* II digestion followed by PCR by using of 72 female patients diagnosed with JIA and 183 female controls, which comprised of newborns (n=91) and children with no history of an autoimmune condition (n=92). A male control (46, XY) was used for complete digestion in the analysis of XCI pattern. We expect to see an association between extremely skewed XCI and female predisposition to JIA.

ÖZET

JÜVENİL İDİYOPATİK ARTRİT HASTALIĞINDA BOZUK X KROMOZOMU ETKİNSİZLEŞTİRİLMESİ

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Jüvenil idiyopatik artrit (JİA) çocukluk çağında görülen en sık romatolojik hastalıktır. Hastalığın görülme sıklığı 7–21/100.000 arasındadır ve genel olarak kız çocuklarında daha sık görülmektedir. Hastalığın oluşumu konusunda çevresel ve genetik etkenler olmak üzere farklı açıklamalar bulunmaktadır. JİA'daki genetik etkenler tam anlamıyla bilinmemektedir. Tek yumurta ikizleri ile yapılan çalışmalar sonucu genetik etkenlerin varlığı saptanmıştır, ancak bu etkenler birçok epigenetik olayları kapsamaktadır. Daha önce kadınlardaki mozaizmin bozulmasının otoimmün hastalıklara neden olabileceği ileri sürülmüştür. Yakın zamanda laboratuarımızda gerçekleştirilen çalışmalar sonucunda bozuk X-etkinsizleştirilmesi ve kadınların otoimmün hastalıklara yatkınlığı arasında bağlantı kurulmuştur. JİA hastalığı da otoimmün bir hastalık olarak bilinmektedir. Bu nedenle bozuk X-etkinsizleştirilmesinin JİA oluşumunda bir etkisi olabileceğini ileri sürüyoruz. 72 hasta ve 183 sağlıklı kontrollerde X-etkinsizleştirilmesi statüsünü belirlemek için androjen reseptörü lokusu metillemeye duyarlı *HpaII* enzimi ile analiz edilmiştir. Kontrol grubu 91 yenidoğan ve 92 sağlıklı çocuktan oluşmuştur. X-etkinsizleştirilmesi analizinde tamamen X-kromozomu kesilmesini göstermek üzere erkek kontrol (46, XY) kullanılmıştır. Burada bozuk X-etkinsizleştirilmesi ve JİA' ya kadın yatkınlığı arasında bir ilişki bulunmasını bekliyoruz.

TO MY MOTHER, TÜRKAN AVŞAR,
FOR HER LOVE AND SUPPORT

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ABBREVIATIONS

ACR	American college of rheumatism
Abs	Antibodies
ANA	Antinuclear antibodies
ASP	Affected sibling pair
bp	base pair
Bisacrylamide	N, N, methylene bisacrylamide
CCP	cyclic citrullinated peptide
ddH_2O	deionized water
DNA	deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ERA	Enthesitis-related arthritis
EtBr	Ethidium bromide
EtOH	Ethanol
EULAR	European league against rheumatism
G6PD	glucose 6-phosphate dehydrogenase
HLA	Human leukocyte antigen
IL	Interleukin
ILAR	International league against rheumatism
JAS	Juvenile ankylosing spondylitis
JCA	Juvenile chronic arthritis
JIA	Juvenile idiopathic arthritis
JRA	Juvenile rheumatoid arthritis

kb	Kilobase
kDa	Kilodalton
MAS	Macrophage Activation Syndrome
$MgCl_2$	Magnesium chloride
mM	Millimolar
ml	Milliliter
µl	Microliter
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PTP	Protein tyrosine phosphatase
RE	restriction enzyme
RF	Rheumatoid factor
SDS	sodium dodecyl sulphate
TAE	tric-acetic acid-EDTA
TCR	T cell receptor
TEMED	N, N, N, N-tetramethyl-1-2, diaminoethane
TNF	Tumor necrosis factor
Xa	Active X
Xi	Inactive X
XCI	X-chromosome inactivation
Xic	X-inactivation center

CHAPTER I: INTRODUCTION

1.1 Juvenile Idiopathic Arthritis

Juvenile idiopathic arthritis (JIA), which is the inflammation (cellular damage) of the synovium (the lining of joints), is the most prevalent pediatric rheumatic disease that is seen in children with onset before 16 years of age. JIA patients have swollen, painful joints (lasting more than six weeks), which may be stiff and difficult to move. The inflammation of the joints may result in damage to the bone and cartilage. This may cause possible changes in bone growth resulting in longer, shorter or bigger affected bones (Figure 1.1) (Cuccurullo, 2004).

Figure 1.1 Leg-length discrepancy in a child with juvenile idiopathic arthritis (Rhodes, 1991)

Arthritis is best described by four major changes in the joints. The most common features of JIA involve the joint are inflammation, contracture, damage and alteration or change in growth. Other symptoms are weakness in muscles and other soft tissues around involved joints. It may also involve organs such as the skin, heart, lungs, liver, spleen, and eyes, producing extra-articular signs and symptoms (Cuccurullo, 2004; Petty *et al*., 2003).

1.1.1. Classification

First proposed in 1994 and later revised in 1997, the term 'juvenile idiopathic arthritis' (JIA) (Petty *et al*., 1998; Petty *et al*., 2003; Petty *et al*., 2004) was used instead of the American term 'juvenile rheumatoid arthritis' (JRA) as defined by American College of Rheumatology (ACR) (Brewer *et al*., 1977) and the European classification 'juvenile chronic arthritis' (JCA) as defined by the European League Against Rheumatism (EULAR) (Wood *et al*., 1978). Because the American and European classifications of the disease were confusing (Table 1.1), it was difficult to use them interchangeably (disease duration is 6 weeks for ACR, while it is 12 weeks for EULAR). In an effort to improve research and treatment, the International League Against Rheumatism (ILAR) has devised a unifying set of international criteria, using the term 'juvenile idiopathic arthritis'. The word 'idiopathic' means 'of unknown cause'. This classification is gaining favor among researchers, but is not yet universally used.

Table 1.1. Comparison of the classification systems of arthritis in children

Classification	ACR	EULAR	ILAR
Designation	JRA	JCA	JIA
Types	Systemic Pauciarticular Polyarticular	Systemic Pauciarticular RF-negative polyarticular RF-positive polyarticular Psoriatic JAS	Systemic Oligoarticular RF-negative polyarthritis RF-positive polyarthritis Psoriatic Enthesitis-related Undefined

ACR=American College of Rheumatology; EULAR=European League against Rheumatism; ILAR=International League of Associations for Rheumatology; JRA= juvenile rheumatoid arthritis; JCA= juvenile chronic arthritis; JIA= juvenile idiopathic arthritis; JAS= juvenile ankylosing spondylitis (Petty *et al*., 2003).

The ILAR classification aims to both unify the previous classifications to minimize international differences in disease definition and to identify clinically homogenous disease subgroups within the term JIA (Petty *et al*., 1998).

1.1.2. Types

According to ILAR, the major subtypes of JIA are oligoarticular JIA, which may be persistent or extended, polyarticular rheumatoid factor (RF)–negative JIA, polyarticular RF-positive JIA, systemic JIA, enthesitis-related arthritis (ERA), psoriatic JIA, or a classification of “other JIA” when the criteria for more than one subtype of JIA or none of the criteria were met (Petty *et al*., 1998).

1.1.2.1. Oligoarticular JIA

Oligoarthritis affects four or fewer joints during the first 6 months of disease. It is the most common type, affecting about 50% of all children with JIA, and mostly seen in females. In the ILAR classification, children who have psoriasis/a family history of psoriasis, a human leukocyte antigen (HLA) B27-associated disease in a first-degree relative, and a positive rheumatoid factor (RF) test are excluded from the oligoarthritis category (Petty *et al*., 1998).

This form of JIA is not seen in adults, and it is characterized by asymmetric arthritis, early age of onset (before 6 years of age), female predominance, high frequency of positive antinuclear antibodies (ANAs), and high risk of iridocyclitis (uveitis), which is an eye inflammation. It is more common in the larger joints, like the knees, ankles or elbows, but can also affect wrists, fingers and toes (Cuccurullo, 2004; Ravelli *et al*., 2007).

According to the ILAR classification, there are two categories in the oligoarthritis subtype: persistent oligoarthritis, in which the disease consists of four or fewer joints, and extended oligoarthritis, in which arthritis extends to more than four joints after the first 6 months of disease (Petty *et al*., 1998; Ravelli *et al*., 2007).

1.1.2.2. Polyarticular JIA

Polyarticular arthritis affects 35% children with JIA, more girls than boys. Symptoms include swelling or pain in 5 or more joints. This kind of JIA usually involves small joints of the hands and feet (Figure 1.2). Large joints, such as knees, wrists, elbows, and ankles are also involved in association with small joints. Additionally, the joints of the neck (cervical spine) and jaw (temporomandibular joints) may also be affected. In addition, a low-grade fever and tiredness may appear. Polyarticular JIA is often symmetrical. There are two types of polyarticular JIA: rheumatoid-factor-positive and rheumatoid-factor-negative polyarthritis (Cuccurullo, 2004; Ravelli *et al*., 2007).

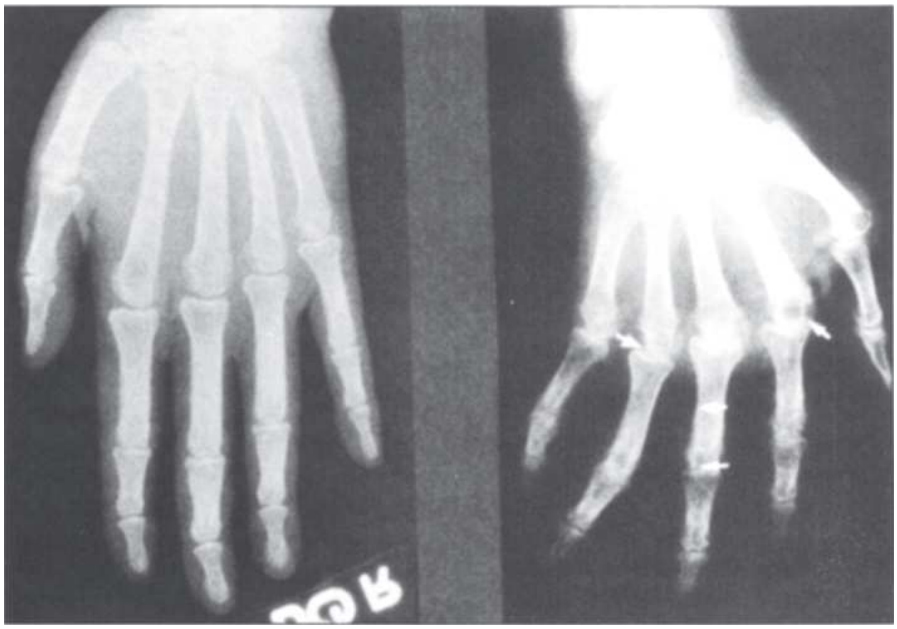


Figure 1.2 Radiographs of normal hand (left) and arthritic hand (right). (Arrows denote loss of normal axial alignment) (Rhodes, 1991)

1.1.2.2.1. Rheumatoid-factor-positive polyarthritis

This disease, comprises 10% of all patients with JIA, and is characterized by age of onset greater than 11 years of age with female predominance (Cuccurullo, 2004). It is the same as adult RF-positive rheumatoid arthritis, except the differences in disease phenotype between children and adults, which are related to the effect of the disease in an individual whose skeleton is still growing. It is mainly seen in adolescent girls (Cuccurullo, 2004; Ravelli *et al*., 2007).

It is characterized as a symmetrical polyarthritis that affects small joints of the hands and feet (Figure 1.3) (Ravelli *et al*., 2007).

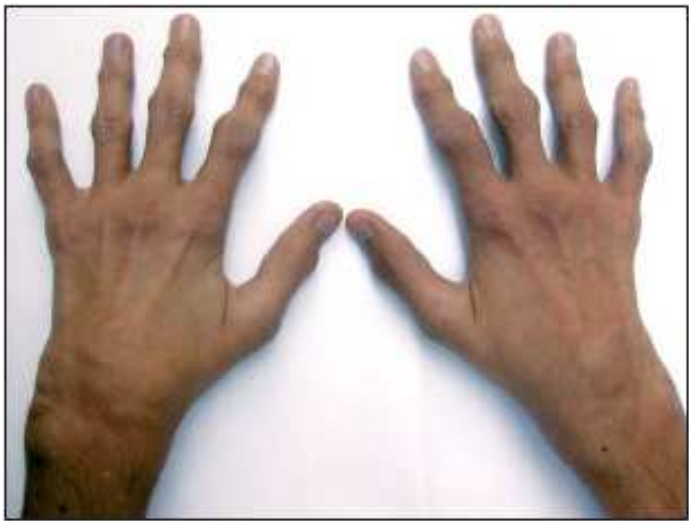


Figure 1.3 Symmetric polyarthritis affecting the metacarpophalangeal, proximal and distal interphalangeal, and radiocarpal joint (Ravelli *et al*., 2007).

1.1.2.2.2. Rheumatoid-factor-negative polyarthritis

This disease is less defined than RF-positive polyarthritis, and is the most heterogeneous subtype (Ravelli *et al*., 2007). It affects 25% of all patients with JIA (Cuccurullo, 2004). There are at least three subsets of RF-negative polyarthritis. The first form resembles early-onset oligoarticular juvenile idiopathic arthritis with the characteristics of asymmetric arthritis, early age of onset, female predominance, frequent positive ANAs, and increased risk of iridocyclitis, except for the number of joints affected in the first 6 months of disease (Martini, 2003; Ravelli *et al*., 2007). The second subset is similar to adult onset RF-negative rheumatoid arthritis, with characteristics of symmetric synovitis of large and small joints, onset in school age, and negative ANA (Ansell, 1987; Ravelli *et al*., 2007). The third form is known as dry synovitis, which shows negligible joint swelling but stiffness, flexion contractures (Figure 1.4). This subset is often poorly responsive to treatment and could follow a destructive progress (Ostrov, 2004; Ravelli *et al*., 2007).

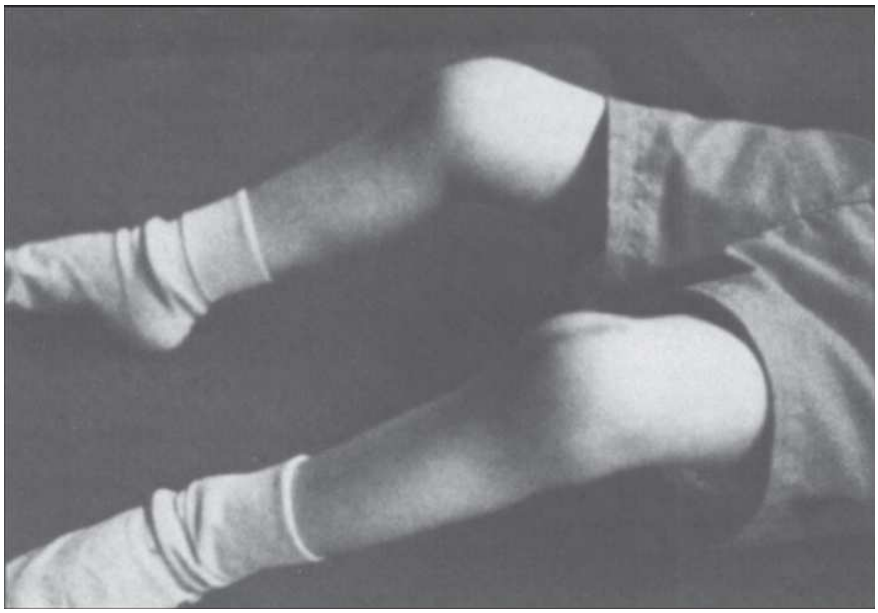


Figure 1.4. Flexion contracture in child with juvenile idiopathic arthritis (Rhodes, 1991).

1.1.2.3 Systemic JIA

It usually begins in early childhood. Researchers sometimes call this Still's disease. This type accounts for about 10-20% of cases of JIA. Systemic arthritis affects both boys and girls almost equally. There may be fever and a rash (Figure 1.5), but joint involvement may not be apparent at first although the child's behavior may indicate joint pain. Fever occurs suddenly and spikes to 39.4°C or higher once or twice daily, usually in the late afternoon. It then rapidly returns to normal or subnormal. It is this discontinuous spiking fever pattern that helps to differentiate the disease from other inflammatory disorders. Other signs and symptoms may include hepatosplenomegaly (enlargement of the liver and spleen), lymphadenopathy (lymph node involvement), pleuritis (or pleurisy -- inflammation of the lining of the lungs or thoracic cavity), pericarditis (inflammation of the sac enclosing the heart), myocarditis (inflammation of the muscular walls of the heart), and nonspecific abdominal pain (Cuccurullo, 2004; Ravelli *et al.*, 2007). Anemia and weight loss may also occur (Martini *et al.*, 1994; Ravelli *et al.*, 2007).

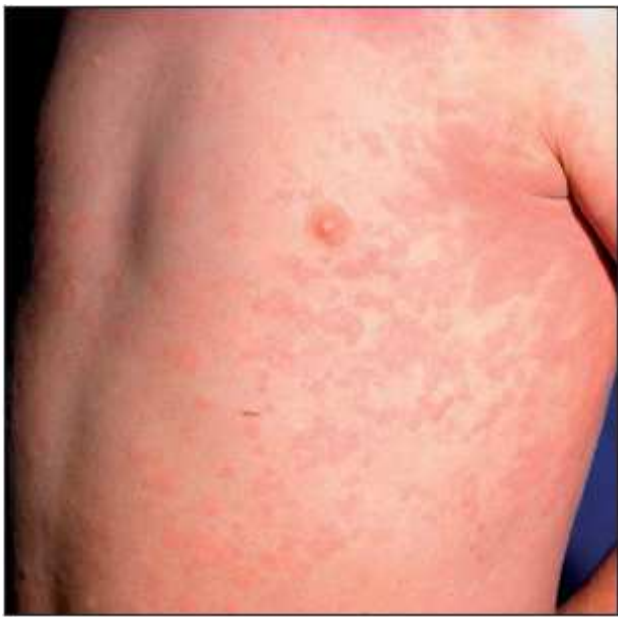


Figure 1.5 Typical rash of systemic-onset disease in an 8-year-old child (Ravelli *et al*., 2007).

1.1.2.4. Enthesitis-related arthritis

Enthesitis is an inflammation of the entheses, the location where a bone has an insertion to a tendon or a ligament. Enthesitis-related arthritis, which is characterized by the association of enthesitis and arthritis, mainly affects male patients after the age of 6 years. Most patients are HLA-B27 positive, and the joints of the lower extremities are affected. Hip involvement is common at disease presentation, resembling oligoarthritis (Petty *et al*., 2001; Petty *et al*., 2003). Enthesitis-related arthritis is often remitting and can be mild. About half of patients have four or fewer joints affected throughout the entire course of the disease (Petty *et al*., 1998; Petty *et al*., 2003; Ravelli *et al*., 2007).

1.1.2.5. Psoriatic arthritis

According to ILAR, in order to diagnose juvenile psoriatic arthritis, arthritis and psoriatic rash need to be present. If a rash is absent, the presence of arthritis and any two of the following: family history of psoriasis in a first-degree relative; dactylitis (sausage-shaped swelling of the fingers and toes, that can be painful); and nail pitting. The symptoms are similar to the subset of RF-negative polyarthritis, and oligoarthritis. The main difference is

that patients with psoriatic arthritis have a greater frequency of dactylitis and of arthritis that affects both small and large joints than do children with oligoarthritis (Petty *et al*., 1998; Ravelli *et al*., 2007).

1.1.2.6 Undifferentiated arthritis

Undifferentiated arthritis does not represent a separate subset, but includes patients who do not satisfy inclusion criteria for any category, or who meet the criteria for more than one (Petty *et al*., 2004; Ravelli *et al*., 2007).

1.1.3. Prevalence and incidence

The incidence and the prevalence of the disease differ among different ethnicity (Table 1.2). The prevalence of a disease in a statistical population is defined as the total number of cases of the disease in the population at a given time, or the total number of cases in the population, divided by the number of individuals in the population, while the incidence is the number of new cases of a disease during a given time interval, usually one year.

Table 1.2 Comparison of incidence and prevalence in different populations

Country	Pre.	Inc.	Reference
Canada	-	4.08/100,000	Malleson *et al*., 1996
Sweden	56/100,000	12/100,000	Andersson *et al*., 1987
Norway	148.1/100,000	22.6/100,000	Moe *et al*., 1998
Finland	-	19.6/100,000	Kunnamo *et al*., 1986
Germany	14.8/100,000	6.6/100,000	von Koskull *et al*., 2001
Costa Rica	34.9/100,000	6.8/100,000	Arguedas *et al*., 1998

Pre= Prevalence, Inc= Incidence.

The frequency of the subtypes differs. There is female predominance, except in the systemic and enthesitis-related arthritis (Table 1.3). In systemic arthritis female-male ratio is equal. In enthesitis-related arthritis, there is male predominance.

Table 1.3 International League of Associations for Rheumatology (ILAR) categories of juvenile idiopathic arthritis

	Frequency*	Onset age	Sex ratio
Systemic arthritis	4–17%	Throughout childhood	F=M
Oligoarthritis	27–56%	Early childhood; peak at 2–4 years	F>>>M
Rheumatoid-factor-positive polyarthritis	2–7%	Late childhood or adolescence	F>>M
Rheumatoid-factor-negative polyarthritis	11–28%	Biphasic distribution; early peak at 2–4 years and later peak at 6–12 years	F>>M
Enthesitis-related arthritis	3–11%	Late childhood or adolescence	M>>F
Psoriatic arthritis	2–11%	Biphasic distribution; early peak at 2–4 years and later peak at 9–11 years	F>M
Undifferentiated arthritis	11–21%	..	..

*Reported frequencies refer to percentage of all juvenile idiopathic arthritis.

(Ravelli *et al*., 2007)

1.1.4. Causes

The cause of JIA is not well-understood. It is believed that JIA is caused by a combination of factors, including genetic factors that make a child's immune system more likely to react inappropriately, an overly active immune system that inappropriately attacks joint tissues, and viral or bacterial infections that may trigger the autoimmune process (Cuccurullo, 2004).

1.1.4.1. Associated Genes

JIA rarely manifests familial occurrence. In the USA total number of affected sibling pairs (ASP) has been estimated to be ~300-400. The National Institute of Arthritis and Musculoskeletal and Skin Diseases has sponsored a research for JIA-affected sibling pairs. Initial analysis showed that 63% of 71 ASPs were concordant for gender and 76% for onset type (Moroldo *et al*., 1997). The study also provided the first estimate of the sibling recurrence risk (λs) for JIA: 15, a value similar to type 1 diabetes. Such a high λs is indicative of a factor shared between sibling, genetic or environment. Researchers from Finland estimated the λs of JIA to be ~20, by using 41 JIA multicase families with 88 affected siblings. In the study, it was calculated that a monozygotic twin of a JIA patient had a relative risk (RR) for developing JIA of about 250 (Savolainen *et al*., 2000; Borchers *et al*., 2006; Glass *et al*., 1999). These data suggest that there is a considerable genetic basis in JIA, but this genetic basis is complex (Reviewed in Borchers *et al*., 2006).

There are both MHC-associated and Non-MHC genes that are found to be associated with JIA. The class I gene, HLA-B27, was the first HLA association found in JIA. It is found that HLA-B27 is a risk factor for oligoarthritis, particularly in older male patients (Rachelefsky *et al*., 1974).

The class II genes HLA-DR1 and HLA-DR4, have been reported to increase risk for polyarthritis. DR4 has a particularly high association with RF–positive polyarticular JIA in older children. Homozygosity for DR4 may carry an increased risk of disease. DR1 is associated with oligoarthritis that converts to a polyarthritis in younger patients, as well as contributing risk for polyarticular disease in older children (Nepom *et al*., 1984; Glass *et al*., 1999).

Table 1.4 lists the non-MHC genes and chromosome regions that have been reported to be associated with JIA (Glass *et al*., 1999). In general, the odds ratios are low and case-control studies have not been as reproducible as HLA association studies (Glass *et al*., 1999).

Table 1.4. Non-HLA genes/loci in juvenile idiopathic arthritis

Polymorphism/chromosome region	Reference
IgA deficiency	Cassidy *et al*., 1977
Complement deficiency	Glass *et al*., 1980
α_1-antitrypsin	Aranaud *et al*., 1977
Amyloid P component	Woo *et al*., 1987
IL-1α promoter	McDowell *et al*., 1995
TNFα/ß	Epplen *et al*., 1995
TCR $V_ß$6.1 null gene	Maksymowych *et al*., 1992
IL-6 promoter	Fishman *et al*., 1998
IL-10	Crawley *et al*., 1999
Chromosome 22	Sullivan *et al*., 1997

IL = interleukin; TNF = tumor necrosis factor; TCR = T cell receptor (Glass *et al*., 1999).

1.1.4.2. Antibodies

JIA is an autoimmune disease, and a wide variety of auto-Abs has been described in patients with JIA (Table 1.5). None of these is specific to JIA and only rheumatoid factor (IgM RF) and antinuclear antibodies (ANA) are routinely used to provide serological support for the diagnosis of JIA.

ANA are detected in ~30–50% of patients with JIA (Berntson *et al*., 2003; Flatø *et al*., 1998; Serra *et al*., 1999; Kotaniemi *et al*., 1999), with prevalence estimates in the individual subtypes ranging from 38% to 85% in oligoarthritis, ~30–50% in polyarthritis and 0–17% in systemic onset disease (Al-Matar *et al*., 2002; Moroldo *et al*., 2004). ANA positivity is one of the most important risk factors for uveitis (Packham *et al*., 2002; Kotaniemi *et al*., 2001), but is not significantly associated with the development of complications and visual outcome (Cabral *et al*., 1994).

One of the specific targets of ANA in JIA is the 45 kDa DEK nuclear antigen (Szer *et al*., 1994; Murray *et al*., 1997), a DNA-binding protein. It was shown to bind specifically to the conserved Y-box regulatory sequences in the human leukocyte antigens (HLA) DQA1*0101 and DQA1*0501 (Adams *et al*., 2003), which is known as a susceptibility allele for oligoarticular JIA (Thomson *et al*., 2002; Borchers *et al*., 2006).

2%- 12% of patients with JIA are positive for IgM RF, including up to 21% of patients with polyarticular disease, 9% of patients with oligoarthritis and 0 - 15% of patients with systemic onset arthritis according to the EULAR and ACR criteria. Rheumatoid factor is an antibody directed against the Fc fragment of IgG. (Minden *et al*., 2000; Kotaniemi *et al*., 1999).

In more recent studies; an ELISA, based on a cyclic citrullinated peptide (CCP) for detection of antibodies against citrullinated proteins in JIA patients, was used. Two of the studies reported significantly high frequencies of anti-CCP in RF-positive polyarthritis patients (73% and 57%, respectively) (van Rossum *et al*., 2003; Ferucci *et al*., 2005; Borchers *et al*., 2006). In a study, in which 3 synthetic citrullinated peptides and 2 different ELISA kits were used, frequencies of anti-CCP antibodies of up to 77% in patients with JIA overall, 93% in RF-negative polyarthritis, 84% in oligoarthritis and 62% in systemic arthritis patients were reported (Low *et al*., 2004; Borchers *et al*., 2006).

Table 1.5 Antibodies described in JIA patients

Antibodies	Reference
ANA	Berntson *et al*., 2003; Flatø *et al*., 1998; Serra *et al*., 1999; Kotaniemi *et al*., 1999
RF	Minden *et al*., 2000; Kotaniemi *et al*., 1999
Antikeratin, Antifilaggrin, Anticitrullinated fibrin, Anti-Sa	Nesher *et al*., 1992; Gabay *et al*., 1993; Hromadnikova *et al*., 2001
Anti-CCP	van Rossum *et al*., 2003; Ferucci *et al*., 2005; Low *et al*., 2004

ANA= antinuclear antibodies; RF= rheumatoid factor; Sa=citrullinated vimentin; CCP=cyclic citrullinated peptide (Borchers *et al*., 2006).

1.2 Autoimmunity

Autoimmunity is the failure of an organism to recognize its own constituent parts (down to the sub-molecular levels) as "self", which results in an immune response against its own cells and tissues. Any disease that results from such an aberrant immune response is termed an autoimmune disease (Janeway *et al*., 2005).

All individuals are tolerant of their own potentially antigenic substances, and failure of self tolerance is the fundamental cause of autoimmunity. The mechanisms of self tolerance have been worked out in considerable detail in animal models, and are best understood for $CD4^+$ T cells (Figure 1.6). Self tolerance can be divided into central tolerance and peripheral tolerance. In central tolerance, immature lymphocytes that happen to recognize self antigens in generative lymphoid organs (the bone marrow for B cells and the thymus for T cells) die by apoptosis; in peripheral tolerance, mature self-reactive lymphocytes encounter self antigens in peripheral tissues and are killed or shut off. The principal mechanisms of peripheral tolerance are anergy (functional unresponsiveness), deletion (apoptotic cell death), and suppression by regulatory T cells.

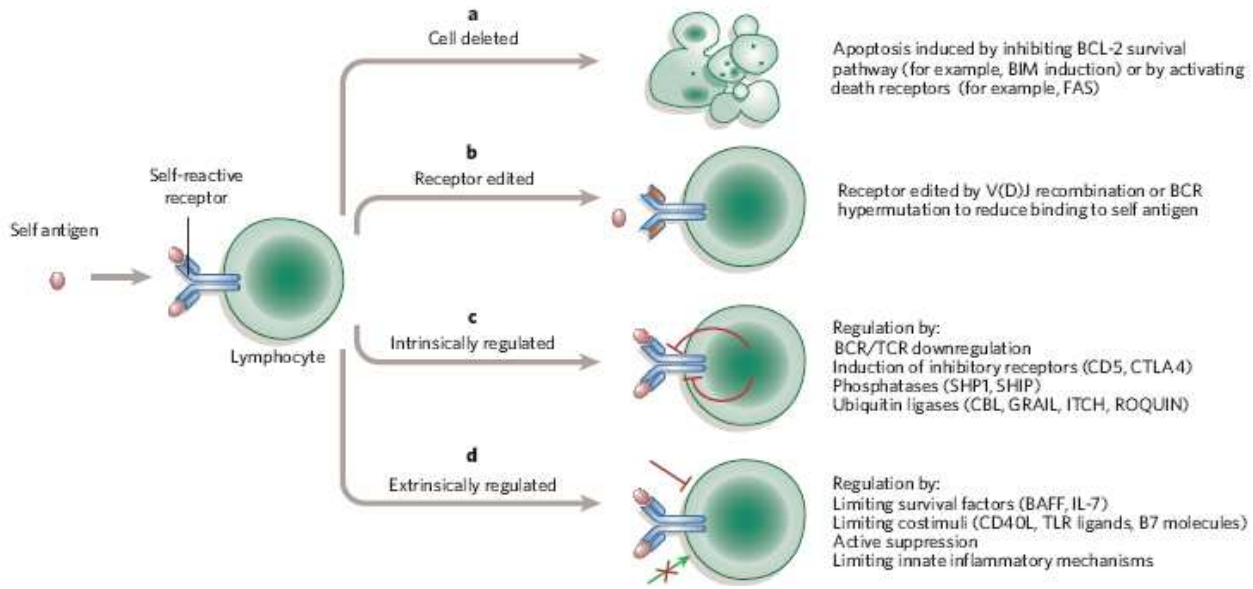


Figure 1.6 Self tolerance (Goodnow *et al*., 2005). a, The cell is deleted through induction of cell death. b, The receptor is edited to one that is less self-reactive. c, Biochemical or gene-expression changes intrinsically dampen the self-reactive receptor's ability to activate the cell. d, The ability of self-reactive cells or antibody to cause autoimmunity is limited by using extrinsic suppression and by limiting essential growth factors, costimuli and inflammatory mediators.

1.2.1 Cause of Autoimmunity

Autoimmune diseases develop when self-reactive lymphocytes escape from tolerance and are activated. Although the mechanisms by which this occurs are not entirely known, autoimmunity is thought to result from a combination of genetic variants, acquired environmental triggers such as infections, and stochastic events (Janeway *et al*., 2005).

1.2.1.1. Genes associated with autoimmunity

1.2.1.1.1. *AIRE*

AIRE (autoimmune regulator) was identified as the gene that is mutated in autoimmune polyendocrine syndrome (APS-1) — a disorder that manifests as autoimmune attack against multiple endocrine organs, the skin and other tissues (Bjorses *et al*., 1998).

The mouse homologue of the gene has been knocked out, and the AIRE protein shown to be responsible for the thymic expression of some antigens that are expressed at high levels in different peripheral tissues. In the absence of thymic expression, T cells specific for these antigens escape negative selection (central tolerance), enter the periphery and attack the target tissues (Anderson *et al*., 2002; Liston *et al*., 2003).

1.2.1.1.2. *CTLA4*

Cytotoxic T lymphocyte antigen 4 (CTLA4; CD152) is an inhibitory receptor expressed by T cells that recognizes the costimulatory molecules B7-1 (CD80) and B7-2 (CD86), the ligation of which shuts off T-cell responses and promotes long-lived anergy (Salomon and Bluestone, 2001). CTLA4 works by competitively blocking the engagement of the activating receptor CD28 (by CD80 or CD86), and by transducing inhibitory signals; the latter probably involves tyrosine and serine/threonine phosphatase activation (Baroja *et al*., 2002).

1.2.1.1.3. *FOXP3*

FOXP3 (encoding a transcription factor of the forkhead family) is a striking example of a gene whose role in autoimmunity has been revealed by the confluence of animal studies and studies of a quite rare human disease. $CD4^{+}CD25^{+}$ regulatory T cells, now established as major controllers of immune responses to self and other antigens (Sakaguchi, 2004), were shown to express high levels of FOXP3. Three groups demonstrated that induced knockout or spontaneous mutation of the mouse *Foxp3* gene led to a systemic autoimmune disease

associated with the absence of $CD4^+CD25^+$ regulatory T cells (Hori *et al*., 2003; Fontenot *et al*., 2003; Khattri *et al*., 2003).

1.2.1.1.4. *PTPN22*

PTPN22 gene maps to chromosome 1p13.3–p13.1 and encodes a lymphoid specific phosphatase (Lyp). Lyp is an intracellular PTP and physically bound through proline-rich motif to the SH3 domain of the Csk kinase, which is an important suppressor of kinases that mediate T-cell activation (Cohen *et al*., 1999). Recently, it was shown that PTPN22 1858C->T SNP may play role in autoimmunity (Bottini *et al*., 2004; Begovich *et al*., 2004). The PTPN22 1858C->T SNP changes the amino acid at position 620 from an arginine (R) to a tryptophan (W) and disrupts the interaction between Lyp and Csk, avoiding the formation of the complex and, therefore, the suppression of T-cell activation (Cloutier *et al*., 1999).

1.2.1.2. Molecular Mimicry

Molecular mimicry is defined as the theoretical possibility that sequence similarities between foreign and self-peptides are sufficient enough to result in the cross-activation of autoreactive T or B cells by pathogen-derived peptides (Janeway *et al*., 2005). Upon the activation of B or T cells, it is believed that these “peptide mimic” specific T or B cells can cross-react with self-epitopes, thus leading to autoimmunity (Kohm *et al*., 2003). Assuming five to six amino acid residues are used to induce a monoclonal antibody response, the probability of 20 amino acids occurring in six identical residues between two proteins is 20^6 or 1 in 64,000,000. However, there has been evidence shown and documented of many molecular mimicry events (Oldstone, 1998).

1.2.1.3. Female Predominance in autoimmunity

1.2.1.3.1. Hormones

In most of the autoimmune diseases, such as autoimmune thyroiditis, systemic lupus erythematosus, and scleroderma there is female predominance, 3-10 fold more affected females (Whitacre, 2001). There are several explanations for this predominance. One of them is sex hormones. (Lockshin, 2002). The inhibitory effects of sex steroids on autoimmune diseases were initially demonstrated in experimental autoimmune thyroiditis induced in guinea pigs and rats by thyroid extract adjuvant administration (Kappas *et al*., 1963). Both testosterone and estrogen at moderately high doses suppressed autoimmune thyroiditis in guinea pigs. Similar effects of testosterone, but not estrogen, were noted in autoimmune thyroiditis induced in rats.

1.2.1.3.2 Chimerism

Another possible explanation for the female predominance in autoimmune diseases is chimerism. Microchimerism resulting from transplacental cells (cell passage from child to mother, or in some instances, mother to child) was considered responsible for autoimmune diseases, including SSc (Mullinax, 1993, Nelson,1996). Fetal DNA and cells were identified in some women with SSc (Nelson, 1996; Mullinax *et al*., 1996; Nelson *et al*., 1998; Artlett *et al*., 1998), raising the possibility that microchimerism play a role in autoimmune diseases. Although these findings are really interesting, microchimerism in SSc could be secondary to the underlying disease, because it offers no explanation for the occurrence of the disease in men or in women who have had no children (Welsh, 1998).

1.2.1.3.3. Skewed XCI

Disturbed X-inactivation is another explanation for the female predominance in autoimmune diseases (Kast, 1977; Stewart, 1998; Chitnis *et al*., 2000). As a result of X-inactivation, the X-chromosome inherited from either parent is silenced at random, and normal women are thus a mosaic of 2 cell populations. Therefore, it is reasonable that skewed

XCI could lead to the escape of X-linked self antigens from presentation in the thymus or in other peripheral sites that are involved in tolerance induction, and loss of T cell tolerance. High frequency of skewed X-inactivation has been observed in breast and ovarian cancers (Kristiansen *et al*., 2002, Buller *et al*., 1999), and in women with recurrent spontaneous abortions (Lanasa *et al*., 1999, Sangha *et al*., 1999). Recently it was shown that there are high numbers of scleroderma patients that have extremely skewed inactivation in the peripheral blood (Ozbalkan *et al*., 2005). Also, in AITD patients, extremely skewed inactivation was observed in peripheral blood (Ozcelik *et al*., 2006).

1.3 X-Inactivation

1.3.1 History

In 1961 Mary Lyon proposed a hypothesis to explain several unexpected results in her analysis of mutations affecting the coat color of female mice. She suggested that only one of the two X-chromosomes functioned in each cell of a female, and the other became inactive; because either parental chromosome could be inactive, females would be mosaics (Lyon, 1961). She also suggested that the X-inactivation event occurred early in development. Therefore, each cell clone formed large patches of different color.

At that time, Ernest Beutler made a similar proposal to explain that females inactivate one X-chromosome in order to maintain dosage parity with the single X-chromosome in males (dosage compensation). Beutler and colleagues formulated the XCI hypothesis using studies of the human X-chromosome gene glucose 6-phosphate dehydrogenase (G6PD) (Beutler *et al*., 1962). They found that in females, G6PD activity was not twice that of males and postulated a dosage compensation mechanism. Using a mixture of male cells with deficient G6PD activity and normal G6PD activity, Beutler and colleagues measured G6PD activity (by glutathione stability) and compared it to the response of female erythrocytes. They concluded that intermediate activity in females was probably due to the same mechanism as in the mixture of male normal and G6PD activity deficient erythrocytes.

After these proposals, a hypothesis came from Ohno, Hauschka, and Makino who demonstrated, first in mice then in humans, that Barr bodies (Barr *et al*., 1949) did not consist

of portions of two X-chromosomes in opposition to each other. Rather, each Barr body was a single X-chromosome (Ohno *et al*., 1960, Ohno *et al*., 1961). Until that time, many believed that the Barr bodies, which were called sex chromatin body at that time, were structures formed by the crossing of the two X-chromosomes in the cell.

1.3.2. Mechanism

Normally, in eutherian mammals, X-inactivation is a random process. By contrast, in marsupials, the X-chromosome coming from the father is always inactivated (Cooper *et al*., 1971). More recently, it was shown that eutherian mammals also have imprinted XCI, as marsupials. But this is limited to extra-embryonic tissues-the placenta (Figure 1.7) (Takagi and Sasaki, 1975). Mouse cells undergo an early, imprinted inactivation of the paternally-derived X-chromosome in four-cell stage embryos. The extraembryonic tissues (which give rise to the placenta and other tissues supporting the embryo) retain this early imprinted inactivation, and thus only the maternal X-chromosome is active in these tissues. In the early blastocyst, this initial, imprinted X-inactivation is reversed in the cells of the inner cell mass (which give rise to the embryo), and in these cells both X-chromosomes become active again. Each of these cells then independently randomly inactivates one copy of the X-chromosome. This inactivation event is irreversible during the lifetime of the cell, so all the descendants of a cell which inactivated a particular X-chromosome will also inactivate that same chromosome. This leads to mosaicism. X-inactivation is reversed in the female germline, so that all ova contain an active X-chromosome.

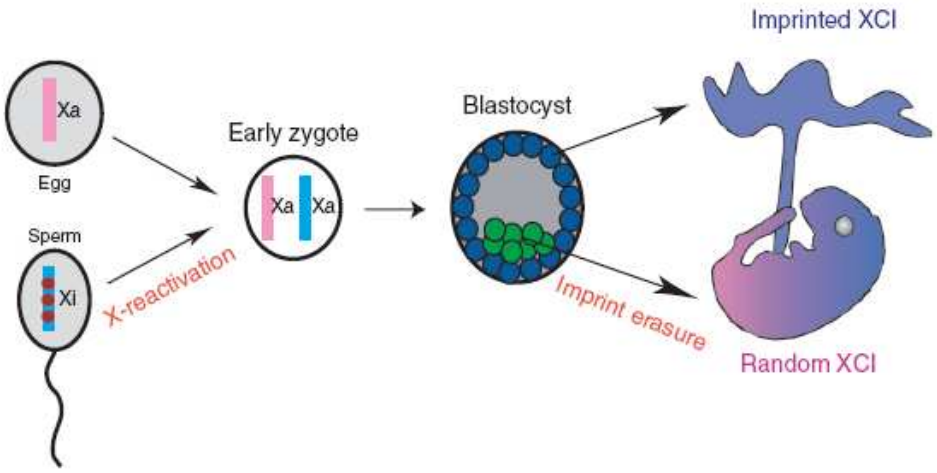


Figure 1.7 Imprinted XCI (Huynh and Lee, 2004).

Random X-inactivation has often been described as a multistep process involving choice of the active X-chromosome (Xa), initiation and spread of silencing on the inactive X-chromosome (Xi), and subsequent maintenance of the Xi's silent state (Chow *et al*., 2005).

Sequences at the *X-inactivation center* (*XIC*), present on the X-chromosome, control the silencing of the X-chromosome. The hypothetical blocking factor is predicted to bind to sequences within the *XIC* (Russell, 1963; Therman *et al*., 1974).

In 1991 a gene located within the *XIC*, the *Xi-specific transcript* was discovered (Borsani *et al*., 1991; Brockdorff *et al*., 1992; Brown *et al*., 1992, Brown, 1991). *XIST* is a gene transcribed from the Xi and not from Xa in somatic cells. No significant open reading frame has been identified, suggesting that *XIST* does not encode a protein. *Xist* has subsequently been shown to be the pivotal player in choice of which X-chromosome remains active, and in the spread of silencing on the future Xi (Marahrens *et al*., 1998). The *Xist* RNA works only in *cis*; that is, on the chromosome that made it.

In 1999, several groups reported the identification of antisense transcription through the *Xist* locus in embryonic stem cells. The transcript, named *Tsix* in recognition of it being antisense to *Xist*, was found to initiate at a major transcription start site 13 kb downstream of the *Xist* 3' end, and to extend across *Xist* into its promoter region. Subsequently, a minor *Tsix* promoter has been identified, and mature *Tsix* transcripts of up to 4 kb have been shown to be

produced by splicing. Like *Xist*, *Tsix* has no significant open reading frame and is not thought to encode a protein (Lee *et al*., 1999).

In general, the women are mosaics of inactive X-chromosome. In some cases, skewed XCI might occur. There are two main reasons of skewed XCI: primary and secondary. A mutation in Xic (X-inactivation-center), or in XIST (X-inactive-specific transcript) is primary cause. The secondary causes are deleterious X-linked mutations, X-chromosome rearrangements, aging, twinning or monoclonal expansion of cells (reviewed by Brown, 1999).

How skewed XCI could lead to self recognition failure is not well understood in a hypothesis, it is thought that skewed XCI can yield a situation in which self-antigens on one X-chromosome may fail to be expressed at sufficiently high levels in the thymus, or in other peripheral sites that are involved in tolerance induction, but may yet be expressed with a high frequency in other peripheral tissues and blood cells. Theoretically, some females may be predisposed to express X-linked antigens in the periphery to which they have been insufficiently tolerized (Brix *et al*., 2005).

1.4. Aim and Strategy

Most of the autoimmune diseases have high female predominance (Whitacre, 2001). Although the female prevalence is often attributed to the effect of estrogen, it is stated that other sex differences might have as much or more relevance to autoimmune disease, that is X-inactivation (Stewart, 1998). Recently, it has been shown that high proportion of scleroderma and AITD patients has extremely skewed X-inactivation in their blood cells (Ozbalkan *et al*., 2005; Ozcelik *et al*., 2006).

JIA is an autoimmune disease, with unknown cause. Like other autoimmune diseases it has female predominance. Here we hypothesize that skewed XCI might play a role in the pathogenesis of JIA. In order to test our hypothesis, we analyzed the methylation status of a highly polymorphic CAG repeat in the androgen receptor (AR) gene. In this study we used JIA patients within the subgroups that have female predominance: oligoarthrits and polyarthritis.

CHAPTER II: MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Patient and Control samples

JIA patients were referred to Bilkent University, Faculty of Science, Molecular Biology and Genetics Department (Ankara, Turkey) by collaborating physicians at Hacettepe University, Faculty of Medicine, Department of Pediatrics, Pediatric Nephrology Unit (Ankara, Turkey). The patients were diagnosed to have oligoarticular or polyarticular JIA. Blood samples were collected in tubes containing EDTA, with the consent forms signed.

2.1.2. Primers

The primers used in polymerase chain reaction (PCR) were synthesized by IONTEK (Bursa, Turkey).

The primer sequences are: primer 1, 5'- GTCCAAGACCTACCGAGGAG -3';
primer 2, 5'- CCAGGACCAGGTAGGCTGTG -3'

2.1.3. Enzymes

Taq DNA polymerases were supplied from MBI Fermentas Inc. (Amherst, NY, USA). *Rsa*I and methylation sensitive *Hpa*II was supplied from Fermentas, Amh, NY, USA.

2.1.4. Thermal cyclers

For PCR reactions, the thermal cycler The GeneAmp System 9600 (Perkin-Elmer, USA) was used.

2.1.5. Chemicals, and kits

Table 2.1. Chemicals, reagents, and kits used in the experiments

Reagent	**Supplier**	**Used for**
Agarose	Basica LE, EU	Agarose Gel electrophoresis
Bisacrylamide	Sigma, St. Louis, MO, USA	Polyacrylamide Gel Electrophoresis
Bromophenol Blue	Sigma, St. Louis, MO, USA	Gel Electrophoresis
Ethanol EtOH	Merck, Frankfurt, Germany	
Ethidium Bromide EtBr	Sigma, St. Louis, MO, USA	Gel Electrophoresis
Proteinase K	Appligene-Oncor, USA	Nucleic Acid Extraction
TEMED	Carlo Erba, Milano, Italy	Polyacrylamide Gel Electrophoresis
APS	Carlo Erba, Milano, Italy	Polyacrylamide Gel Electrophoresis
EDTA pH 8.0	Carlo Erba, Milano, Italy	TAE
Nucleospin® Blood kit	Macherey-Nagel Inc., PA, USA	DNA isolation

2.1.6. Standard solutions and buffers

1X TAE (Tris-acetic acid-EDTA):	40mM Tris-acetate, 2 nM EDTA, pH 8.0
Ethidium bromide:	10mg/ml in water (stock solution) 30 ng/ml (working solution)
Agarose Gel Loading Buffer (6X):	15% ficoll 0.05% bromophenol 0.05% xylene cyanol
Acrylamide:Biacrylamide Stock Solution (%30):	29.5 gr acrylamide 0.44 gr bisacrylamide 100 ml with ddH_2O

2.1.7. Nucleic acids

DNA marker, pUC Mix8 was supplied from MBI Fermentas, Amh, NY, USA

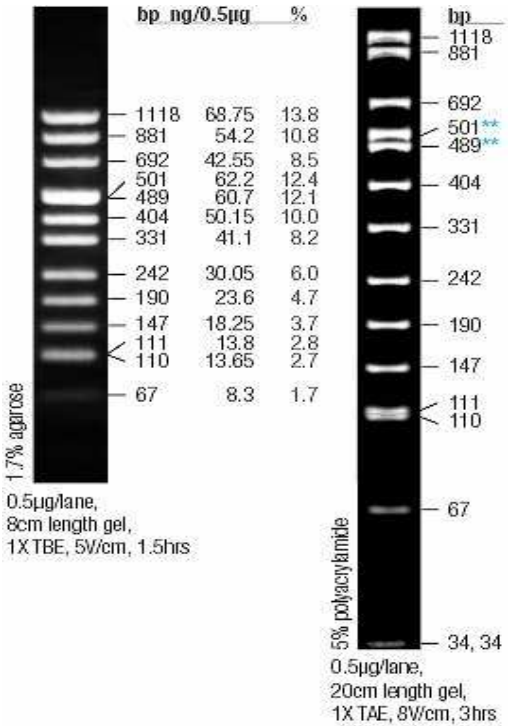


Figure 2.1. Sizes of the fragments of PUC mix marker, 8 and appearance on both agarose and polyacrylamide gel electrophoresis (MBI Fermentas web site)

2.2 METHODS

2.2.1. Sample collection

Blood was obtained from JIA patients and controls, and collected in tubes containing EDTA. They were divided into 1 ml aliquots in 1.5 ml eppendorf tubes. 200 µl of blood was used for DNA isolation; the remaining bloods were stored at -80^{o}C for later use.

2.2.2. Determination of X-chromosome inactivation status

For determination of XCI status, HUMARA assay was used (Figure 2.2). Isolated DNAs were digested by methylation-sensitive enzyme *Hpa*II. After incubation with *Hpa*II, the sites on the active X-chromosome (checkered) will be cleaved, since they are unmethylated; the sites on the inactive X will not be cleaved, since they are methylated. Amplification by PCR between these primers will only yield a product from the uncleaved inactive X-chromosome. The X-inactivation patterns are therefore assessed in a female who is informative at the CAG repeat. The maternal and paternal alleles are resolved using PAGE (polyacrylamide gel electrophoresis) The HUMARA alleles are shown as single bands, for graphic clarity. In practice, each allele is represented by two major and two or more minor bands (Allen *et al*., 1992).

A 280-bp PCR amplification unit including the flanking *Hpa*II sites and the trinucleotide repeat element (nucleotides 229-508) was developed for the human androgen-receptor locus (Figure 2.3).

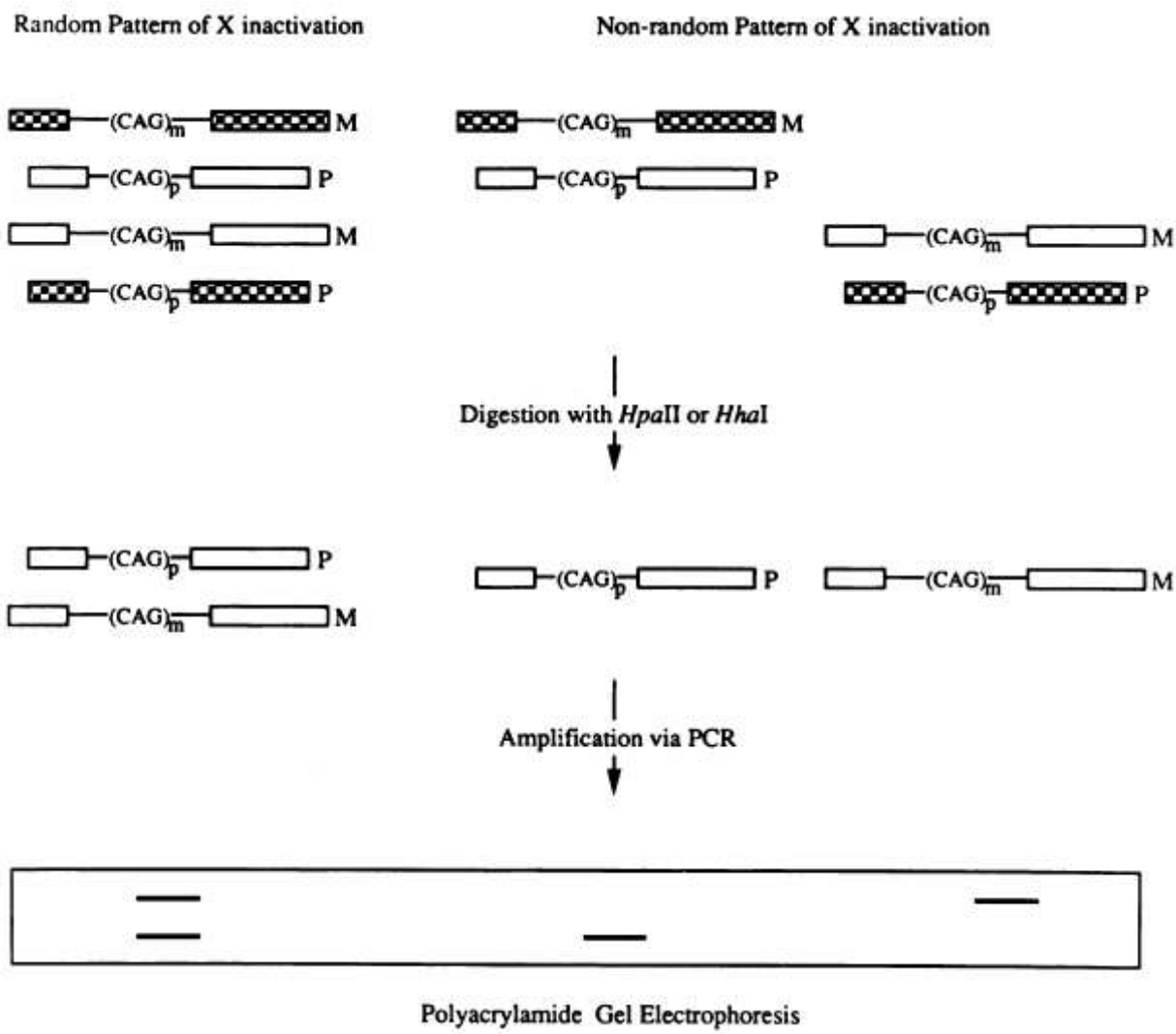
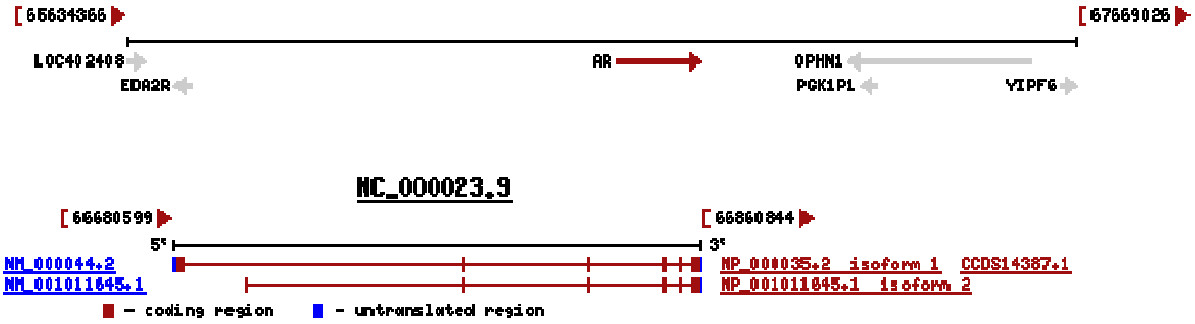


Figure 2.2. Analysis of X-chromosome inactivation patterns by HUMARA assay. The flow diagram illustrates expected results from DNA isolated from cell populations showing either random (left) or nonrandom (right) X-inactivation patterns. M and P = maternal and paternal X-chromosomes, respectively; (CAG)m and (CAG)p = allele associated with the polymorphic CAG repeat on the maternal and paternal X-chromosomes, respectively. (Allen *et al*., 1992).

Chromosome: X; **Location:** Xq11.2-q12 **Gene:** AR



>ref|NC_000023.9|NC_000023:66680599-66860844 Homo sapiens chromosome X, reference assembly

GGGAAAAAGGGCCGAGCTAGCCGCTCCAGTGCTGT▼ACAGGAGCCGAAGGGACGCACCACGCCAGCCCCA

***Rsa*I**

GCCC▼GGCTCCAGCGACAGCCAACGCCTCTTGCAGCGCGGCGGCTTCGAAGCCGCCGCCC▼GGAGCTGCC

HpaII **HpaII**

CTTTCCTCTTCGGTGAAGTTTTTAAAAGCTGCTAAAGACTCGGAGGAAGCAAGGAAAGTGCCTGGTAGGA
CTGACGGCTGCCTTTGTCCTCCTCCTCTCCACCCCGCCTCCCCCCACCCTGCCTTCCCCCCCTCCCCCGT
CTTCTCTCCCGCAGCTGCCTCAGTCGGCTACTCTCAGCCAACCCCCCTCACCACCCTTCTCCCCACCCGC
CCCCCCGCCCCCGTCGGCCCAGCGCTGCCAGCCCGAGTTTGCAGAGAGGTAACTCCCTTTGGCTGCGAGC
GGGCGAGCTAGCTGCACATTGCAAAGAAGGCTCTTAGGAGCCAGGCGACTGGGGAGCGGCTTCAGCACTG
CAGCCACGACCCGCCTGGTTAGGCTGCACGCGGAGAGAACCCTCTGTTTTCCCCCACTCTCTCTCCACCT
CCTCCTGCCTTCCCCACCCCGAGTGCGGAGCCAGAGATCAAAAGATGAAAAGGCAGTCAGGTCTTCAGTA
GCCAAAAAACAAAACAAACAAAAACAAAAAAGCCGAAATAAAAGAAAAAGATAATAACTCAGTTCTTATT
TGCACCTACTTCAGTGGACACTGAATTTGGAAGGTGGAGGATTTTGTTTTTTTCTTTTAAGATCTGGGCA
TCTTTTGAATCTACCCTTCAAGTATTAAGAGACAGACTGTGAGCCTAGCAGGGCAGATCTTGTCCACCGT
GTGTCTTCTTCTGCACGAGACTTTGAGGCTGTCAGAGCGCTTTTTGCGTGGTTGCTCCCGCAAGTTTCCT
TCTCTGGAGCTTCCCGCAGGTGGGCAGCTAGCTGCAGCGACTACCGCATCATCACAGCCTGTTGAACTCT
TCTGAGCAAGAGAAGGGGAGGCGGGGTAAGGGAAGTAGGTGGAAGATTCAGCCAAGCTCAAGGATGGAAG
TGCAGTTAGGGCTGGGAAGGGTCTACCCTCGGCCGCCGTCCAAGACCTACCGAGGAGCTTTCCAGAATCT

RS6 (Forward Primer)

GTTCCAGAGCGTGCGCGAAGTGATCCAGAACCC▼GGGCCCCAGGCACCCAGAGGCCGCGAGCGCAGCACC

HpaII

TCCC▼GGCGCCAGTTTGCTGCTGCTGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA

HpaII ***CAG Repeats***

GCAGCAGCAGCAGCAGCAGCAGCAAGAGACTAGCCCCAGGCAGCAGCAGCAGCAGCAGGGTGAGGATGGT

TCTCCCCAAGCCCATCGTAGAGGCCCCACAGGCTACCTGGTCCTGGATGAGGAACAGCAACCTTCACAGC

RS7 (Reverse Primer)

CGCAGTCGGCCCTGGAGTGCCACCCCGAGAGAGGTTGCGTCCCAGAGCCTGGAGCCGCCGTGGCCGCCAG
CAAGGGGCTGCCGCAGCAGCTGCCAGCACCTCC▼GGACGAGGATGACTCAGCTGCCCCATCCACGTTGTC

HpaII

CCTGCTGGGCCCCACTTTCCCC▼GGCTTAAGCAGCTGCTCCGCTGACCTTAAAGACATCCTGAGCGAGGC

HpaII

CAGCACCATGCAACTCCTTCAGCAACAGCAGCAGGAAGCAGTATCCGAAGGCAGCAGCAGCGGGAGAGCG
AGGGAGGCCTCGGGGGCTCCCACTTCCTCCAAGGACAATTACTTAGGGGGCACTTCGACCATTTCTGACA
ACGCCAAGGAGTTGTGTAAGGCAGTGTCGGTGTCCATGGGCCTGGGTGTGGAGGCGTTGGAGCATCTGAG
TCCAGGGGAACAGCTTCGGGGGGATTGCATGT▼ACGCCCCACTTTTGGGAGTTCCACCCGCTGTGCGTCC

***Rsa*I**

Figure 2.3 The sequence of AR, exon 1. The region amplified by PCR, the *Hpa*II digestion sites and CAG repeat regions are shown.

2.2.2.1. DNA Isolation

The DNA isolation was carried out from 200 µl bloods via Nucleospin® Blood kit (Macherey-Nagel Inc., PA, USA) according to manufacturer's instructions. The remaining bloods were stored at -80oC for later use.

The concentration of the DNA was checked by spectrophotometric reading and horizontal 1% agarose gel electrophoresis in 1X TAE buffer. The DNA samples were loaded on gel after mixing with 6X loading buffer. 1 µg/ml ethidium bromide was added for visualization. After the run, the DNA samples were visualized with UV transilluminator. The spectrophotometric reading was done in order to check the quality and quantity of the DNA. Ratio of 260/280 reading of 1.8 ± 0.2 was accepted as high quality DNA. In addition, 1% agarose gel electrophoresis verifies that the DNA is high-molecular weight.

2.2.2.2. Restriction Enzyme Digestion

Restriction enzyme digestion was carried out from 5 µl genomic DNA isolated from the bloods in 20 µl reaction volumes in 500 µl tubes. Methylation specific *HpaII* and *Rsa*I enzymes were used for determination of X-inactivation status. The undigested control samples were only digested with *Rsa*I enzyme using the conditions and materials (reaction buffer and BSA) given in the manufacturer's instructions. One unit from each enzyme was used for the reaction. The digestion reactions were incubated at 37^{o}C in the incubator for overnight.

Undigested		*Digested*	
DNA	5.0 µl	DNA	5.0 µl
Buffer	2.0 µl	Buffer	2.0 µl
*Rsa*I	0.2 µl	*Rsa*I	0.2 µl
ddH_2O	12.8 µl	*HpaII*	0.8 µl
		ddH_2O	12.0 µl

2.2.2.3. Polymerase chain reaction (PCR)

7 µl template DNA (100-150 ng) was used in 25 µl PCR reaction containing 1X PCR buffer(10X), 1 µl $MgCl_2$ (1.5 mM), 0.3 µl dNTPs (10 mM), 0.5 µl (20 pmol) from each primer and 0.2 µl *Taq* DNA Polymerase (5U). And ddH_2O. Amplification was done using The GeneAmp System 9600 (Perkin-Elmer, USA) under following conditions:

Initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 sec (denaturation), 58°C for 30 sec (annealing), 72°C for 30 sec (extension) and a final extension at 72°C for 5 min.

2.2.2.4. Agarose gel electrophoresis

PCR products were run in the 1.5% agarose gel by using 1X TAE.

Agarose was completely dissolved in 1X TAE electrophoresis buffer to required percentage in microwave and ethidium bromide was added to final concentration of 30ng/ml.

The samples were loaded onto agarose gel with 1/5 volume of loading buffer. The gel was run in 1X TAE at different voltage and time depending on the size of the fragment at room temperature.

2.2.2.5. Polyacrylamide gel electrophoresis (PAGE)

The working PCR products were run in the 8% Polyacrylamide Gel

8% PAGE

Acrylamide: Bisacrylamide (29:1)	40 ml
10x TAE	15 ml
10% APS	1.5 ml
TEMED	100 µl
ddH_2O	93.5 ml

The polyacrylamide solution was poured into the vertical apparatus and the digests were run at different W, and time, depending on the number of gels in 1X TAE buffer. After the run the gels were stained with EtBr for 10 minutes, washed with ddH_2O for 10 minutes.

2.2.2.6. Densitometric Analysis

Densitometric analysis of the alleles was performed using Multi-Analyst software version 1.1 (Bio-Rad, Hercules, CA). A corrected ratio (CrR) was calculated by dividing the ratio of the predigested sample (upper/lower allele) by the ratio of the nonpredigested sample for normalization of the ratios that were obtained from the densitometric analyses.

CHAPTERIII: RESULTS

3.1. PCR-based X-inactivation study of peripheral blood

Androgen receptor assay was performed in order to determine the XCI patterns in the JIA patients and healthy controls, as explained in the methods section. Methylated inactive X-chromosome is resistant to digestion by methylation specific HpaII enzyme, while unmethylated X-chromosome can be digested. Androgen receptor assay is used for XCI status detection; because there are highly polymorphic triplet repeats adjacent to the methylation site in the androgen receptor, which provide difference in lengths of the alleles. The concentration difference of more than 80% between the two alleles is considered as skewed XCI (Allen *et al*., 1992; Naumova *et al*., 1996).

We studied XCI patterns of 72 JIA female patients, 183 female controls. The control group comprised newborns (n=91) and children with no history of an autoimmune condition (n=92). XCI pattern was informative in 56 of the 72 JIA patients, 52 of 91 newborn and 72 of 92 children healthy controls (Appendices). The individuals, who do not show significant difference between two alleles were considered uninformative since only those whose alleles resolve adequately for densitometric analyses were included in the study. In Figure 3.1, patients with uninformative, random and skewed XCI patterns are shown. Sample 06-15 shows uninformative XCI pattern as there is no significant difference between alleles, while samples 06-16 and 06-17 are informative as both alleles can be seen.

Densitometric analysis is described in the methods section. By densitometric analysis, sample 06-17 was determined as having random XCI pattern, while the sample 06-16 has extremely skewed XCI pattern (Figure 3.1).

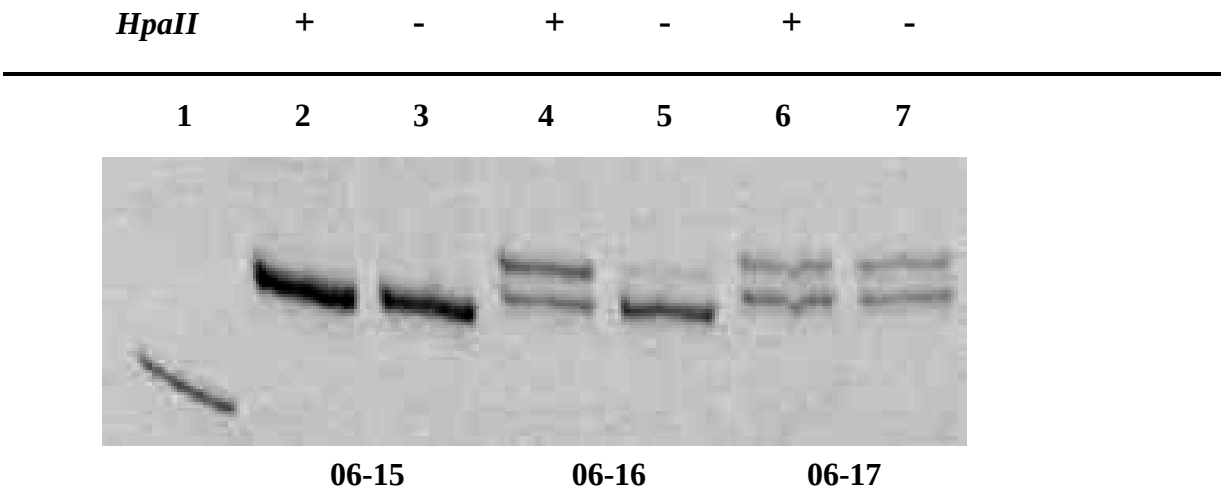


Figure 3.1 X-chromosome inactivation status in JIA patients. Lane 2-3: 06-15; lane 4-5: 06-16; lane 6-7: 06-17. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate random XCI patterns in sample 06-17 (allele ratio 51%:49%), and skewed patterns in sample 06-16 (93%:7%). The sample 06-15 is not informative for the androgen receptor polymorphism. For each sample, DNA was either undigested (−) or digested (+) with the methylation-sensitive restriction enzyme *Hpa*II. Lane 1: Marker (pUC mix 8, 242-bp fragment is visible).

Skewed XCI (>80% skewing) was observed in 14 of the 56 patients (25%) (Table 3.1), and 12 of the 124 controls (9.7%). Extremely skewed XCI, defined as >90% inactivation of one allele, was present in 9 patients (16.1%), and in only 4 controls (3.2%, P=0.002, OR=16.9, 95% CI 6.2-45.8) (Appendices).

Table 3.1 Proportion of the JIA patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewing	
	JIA patients (n=56)	**Controls (n=124)**
90+	9 (16.1)	4 (3.2)
80-89	5 (8.9)	8 (6.5)
70-79	12 (21.4)	28(22.6)
60-69	15 (26.8)	37 (29.8)
50-59	15 (26.8)	47 (37.9)

For comparison by χ^2, P= 0.006 (>80% skewing); P= 0.002 (>90% skewing)

CHAPTER IV: DISCUSSION

A reduction in the sex ratio (male: female) is the characteristic of the most autoimmune diseases (Whitacre, 2001). For several years candidate mechanisms that could be important in pathogenesis have been uncovered. Nowadays, there are some explanations for the female predominance in autoimmune diseases. These include genetic traits associated with autoimmunity (Rioux and Abbas, 2005), pregnancy related microchimerism (Adams and Nelson, 2004), and disturbances of XCI in female subjects (Stewart, 1998).

In this study we demonstrate skewed XCI patterns (>80:20) in peripheral blood mononuclear cells of a significant proportion (25%) of females with JIA. 11.1% of female healthy children control subjects demonstrate skewed X-inactivation patterns, while 7.6% of female newborn control subjects had skewed XCI pattern. When extremely skewed XCI is concerned (>90:10), the results are more distinctive: 16.1% of the JIA patients have extremely skewed XCI, while 2.8% of the healthy children, and 3.8% healthy newborn controls have skewed XCI. When we take all control subjects together, we found the frequency of controls with extremely skewed XCI as 3.2% (OR=16.9 95% CI 6.2-45.8). This is consistent with the findings in the world, which change 1%-6% (OR 4-6) (Chitnis *et al*., 2000; Buller *et al*., 1999; Lanasa *et al*., 1999; Sangha *et al*., 1999). Our results suggest that skewed XCI is associated with the pathogenesis of JIA. Previously in our group, association between skewed XCI and SSc and AITD was shown. To the best of my knowledge, this is the first time that an association between skewed XCI and a pediatric disease is observed.

There are several mechanisms that are used in order to determine XCI pattern, such as protein isoforms and transcription based methods. The exonic polymorphisms, which are used to identify the X-chromosome, are typically non-synonymous mutations. A variety of genes are used to determine XCI status including G6PD (Prchal and Guan, 1993), IDS

(iduronate-2-sulfatase) (Gregg *et al*., 2000; el-Kassar *et al*., 1997), MPP1 (also known as p55) (Luhovy *et al*., 1995), BTK (Bruton tyrosine kinase), and FHL-1 (4.5 LIM domain 1) (Liu *et al*., 2003). In this study we used HUMARA assay, which is based on DNA methylation and tandem CAG repeats. The HUMARA assay is more widely used than protein isoform and transcription based methods because of the variable number of CAG nucleotide repeats allowing most patients to be informative for the assay. In our study we had a limited number of patients, as we used only specific subtypes that have female predominance. Therefore, it is important to have all patients informative for the assay. From 72 patients, 56 were informative for CAG repeats in AR, while 124 of 183 controls were informative. It would be helpful to use the genes above for the patients and controls that are not informative for CAG repeats in AR.

There are mainly two reasons for skewed XCI: primary and secondary. When there is a mutation in *XIST* (X-inactive- specific transcript) and *Xic*, it is called primary cause (Puck, 1998). The secondary causes are deleterious X-linked mutations, X-chromosome rearrangements, aging, twinning, or monoclonal expansion of cells (Brown, 1999). Here we propose that as a secondary cause, a putative lethal mutation on the X-chromosome may result in a cell-survival disadvantage. Cells that carry a putative lethal mutation in their active X do not survive because of the mutation, causing loss of mosaicism.

Because of this survival disadvantage, skewed XCI occurs. The self antigens on the inactive X of these cells are not presented in the thymus or in other peripheral sites. Because of an unknown mechanism, at a later stage of the life, these self antigens encounter the lymphocytes. Therefore, these self antigens are recognized as non-self and cause autoimmunity.

4.1. Future Perspective

In order to prove our loss of mosaicisim hypothesis, we are going to conduct a comprehensive genomic study by using high-density microarray analysis. We searched for all genes and nonsynonymous single nucleotide polymorphisms (SNPs) on the X-chromosome using NCBI and Ensembl databases. After elimination of the redundancies, we identified 2715 nonsyn+syn SNPs, and 7277 intronic SNPs with the heterozygosity value between 0.40

and 0.50. The flanking regions of these SNPs were compiled through the use of an algorithm. At the end, we had 1141 coding synonymous + 1725 coding nonsynonymous + 4 coding not determined, totally 2870 coding SNPs which corresponds to 783 genes. 2802 intronic SNPs which corresponds to 160 genes. With this study, we will analyze copy number variation and allele frequencies of heterozygous genes. For this study, we will use all the autoimmune diseases studied in our lab, including JIA, and healthy controls.

CHAPTER V: REFERENCES

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CHAPTER VII. APPENDICIES

Appendix A The list of JIA patients and their XCI pattern

Number		Sample	XCI Pattern
90+	16.10%		
1	1	06-029	100
2	2	05-069	100
3	3	05-188	95
4	4	06-014	95
5	5	05-180	95
6	6	06-013	95
7	7	06-016	93
8	8	06-030	92
9	9	06-008	90
80-89	8.90%		
10	1	05-179	88
11	2	06-026	84
12	3	06-018	81
13	4	05-064,05-186	80
14	5	06-020	80
70-79	21.40%		
15	1	05-170	78
16	2	06-012	76
17	3	05-071	75

18	4	05-178	75
19	5	06-021	75
20	6	06-052	74
21	7	05-187	73
22	8	05-183	72
23	9	06-009	71
24	10	06-043	71
25	11	05-065	70
26	12	05-063	70
60-69	26.80%		
27	1	05-070	69
28	2	06-025	69
29	3	05-601	64
30	4	05-173	63
31	5	05-182	63
32	6	06-024	62
33	7	06-037	62
34	8	06-044	62
35	9	05-602	61
36	10	06-028	61
37	11	05-073	60
38	12	05-072	60
39	13	05-062	60
40	14	06-051	60
41	15	05-066	60
50-59	26.80%		
42	1	05-168	58
43	2	06-034	57
44	3	05-167	56
45	4	05-169	56
46	5	06-038	56
47	6	05-177	55
48	7	06-019	54

49	8	06-027	53
50	9	06-031	53
51	10	06-048	52
52	11	05-166	51
53	12	05-185, 06-17	51
54	13	06-046	51
55	14	06-045	50
56	15	06-053	50
NI	22.20%		
57	1	05-067	NI
58	2	05-068	NI
59	3	05-171, 06-15	NI
60	4	05-172	NI
61	5	05-174	NI
62	6	05-175	NI
63	7	05-176	NI
64	8	05-181	NI
65	9	05-184	NI
66	10	06-007	NI
67	11	06-022	NI
68	12	06-023	NI
69	13	06-032	NI
70	14	06-033	NI
71	15	06-035	NI
72	16	06-036	NI

Appendix B The list of healthy children controls and their XCI pattern

Number		Sample	XCI Pattern
90+	2.80%		
1	1	05-332	100
2	2	06-072	100
80-89	8.30%		
3	1	05-583	88
4	2	05-370	85
5	3	05-369	85
6	4	06-056	85
7	5	06-071	82
8	6	05-588	82
70-79	22.2		
9	1	06-057	79
10	2	06-070	78
11	3	05-595	75
12	4	06-077	75
13	5	05-587	75
14	6	05-348	75
15	7	05-359	75
16	8	05-599	75
17	9	06-068	75
18	10	05-375	73
19	11	05-372	73
20	12	06-066	73
21	13	05-342	72
22	14	06-059	70
23	15	05-354	70
24	16	06-069	70
60-69	27.80%		
25	1	05-347	68

26	2	05-331	67
27	3	05-585	66
28	4	05-360	65
29	5	05-592	65
30	6	05-357	64
31	7	05-366	64
32	8	05-364	63
33	9	05-596	63
34	10	05-356	62
35	11	05-581	62
36	12	06-074	62
37	13	06-081	62
38	14	05-350	61
39	15	05-374	61
40	16	05-377	61
41	17	06-075	61
42	18	06-061	60
43	19	05-591	60
44	20	05-339	60
50-59	38.90%		
45	1	05-341	58
46	2	05-358	58
47	3	05-367	58
48	4	05-582	58
49	5	05-594	58
50	6	06-058	58
51	7	05-333	57
52	8	05-351	57
53	9	05-346	57
54	10	06-078	56
55	11	06-060	55
56	12	05-337	55
57	13	05-355	55

58	14	05-368	55
59	15	05-586	55
60	16	05-338	55
61	17	05-336	55
62	18	06-067	55
63	19	05-349	54
64	20	05-584	54
65	21	05-329	53
66	22	05-593	53
67	23	05-363	51
68	24	06-080	51
69	25	05-590	50
70	26	05-343	50
71	27	05-598	50
72	28	06-079	50
NI	21.70%		
73	1	05-330	NI
74	2	05-344	NI
75	3	05-345	NI
76	4	05-361	NI
77	5	05-362	NI
78	6	05-365	NI
79	7	05-371	NI
80	8	05-373	NI
81	9	05-376	NI
82	10	05-379	NI
83	11	05-589	NI
84	12	05-600	NI
85	13	06-055	NI
86	14	06-062	NI
87	15	06-065	NI
88	16	06-073	NI
89	17	06-076	NI

90	18	05-353	NI
91	19	05-378	NI
92	20	05-352	NI

Appendix C The list of health newborn children and their XCI pattern

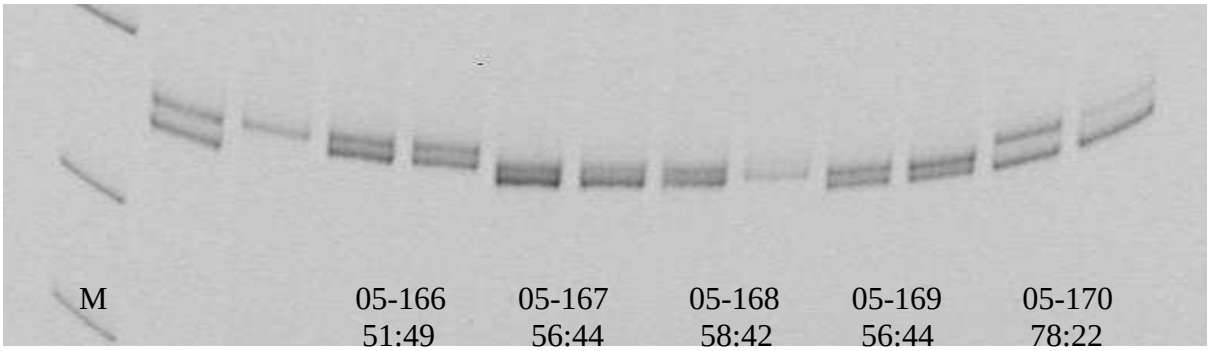
Numbers	Samples		XCI Pattern
90+	3.30%		
1	1	06-830	100
2	2	06-848	99
80-89	3.80%		
3	1	06-863	82
4	2	06-865	80
70-79	23.10%		
5	1	06-837	78
6	2	06-850	78
7	3	06-875	78
8	4	06-868	77
9	5	06-896	77
10	6	06-823	75
11	7	06-824	75
12	8	06-801	75
13	9	06-805	75
14	10	06-893	72
15	11	06-843	71
16	12	06-844	70
60-69	32.70%		
17	1	06-797	68
18	2	06-889	68
19	3	06-870	67
20	4	06-851	66
21	5	06-862	66
22	6	06-838	65
23	7	06-900	65
24	8	06-882	65
25	9	06-827	64
26	10	06-881	63
27	11	06-877	62

28	12	06-884	62
29	13	06-820	61
30	14	06-847	61
31	15	06-811	61
32	16	06-826	60
33	17	06-804	60
50-59	36.50%		
34	1	06-891	59
35	2	06-810	58
36	3	06-831	58
37	4	06-869	58
38	5	06-864	57
39	6	06-883	57
40	7	06-871	55
41	8	06-836	55
42	9	06-873	54
43	10	06-888	54
44	11	06-846	53
45	12	06-845	52
46	13	06-878	52
47	14	06-860	51
48	15	06-876	50
49	16	06-832	50
50	17	06-874	50
51	18	06-880	50
52	19	06-822	50
NI	42.90%		
53	1	06-840	NI
54	2	06-839	NI
55	3	06-814	NI
56	4	06-800	NI
57	5	06-802	NI
58	6	06-803	NI

59	7	06-807	NI
60	8	06-809	NI
61	9	06-812	NI
62	10	06-813	NI
63	11	06-815	NI
64	12	06-821	NI
65	13	06-825	NI
66	14	06-828	NI
67	15	06-829	NI
68	16	06-841	NI
69	17	06-842	NI
70	18	06-849	NI
71	19	06-861	NI
72	20	06-866	NI
73	21	06-867	NI
74	22	06-872	NI
75	23	06-879	NI
76	24	06-885	NI
77	25	06-886	NI
78	26	06-887	NI
79	27	06-890	NI
80	28	06-892	NI
81	29	06-894	NI
82	30	06-895	NI
83	31	06-897	NI
84	32	06-898	NI
85	33	06-899	NI
86	34	06-799	NI
87	35	06-808	NI
88	36	06-816	NI
89	37	06-817	NI
90	38	06-819	NI
91	39	06-818	NI

Appendix E The PAGE figures of XCI patterns of JIA patients

GEL1



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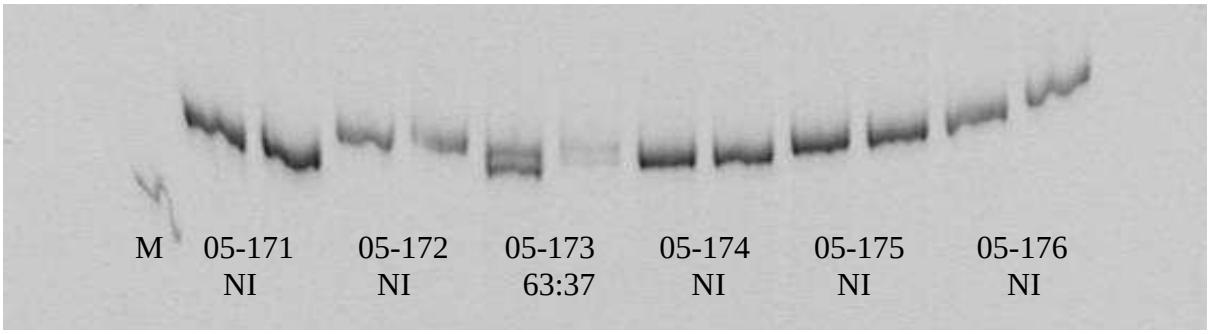
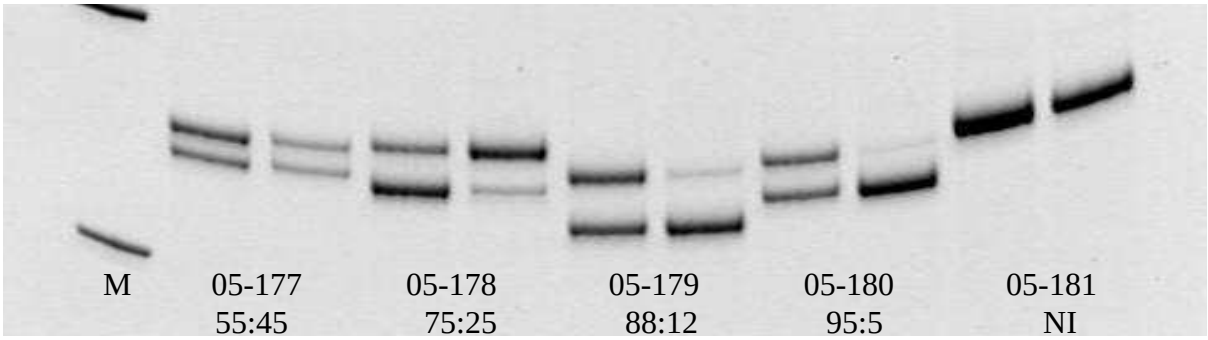
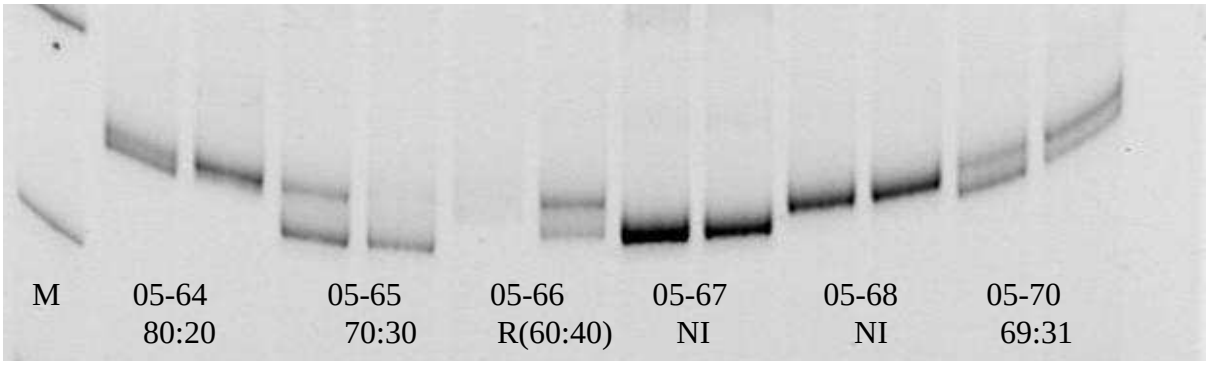


GEL3



GEL4



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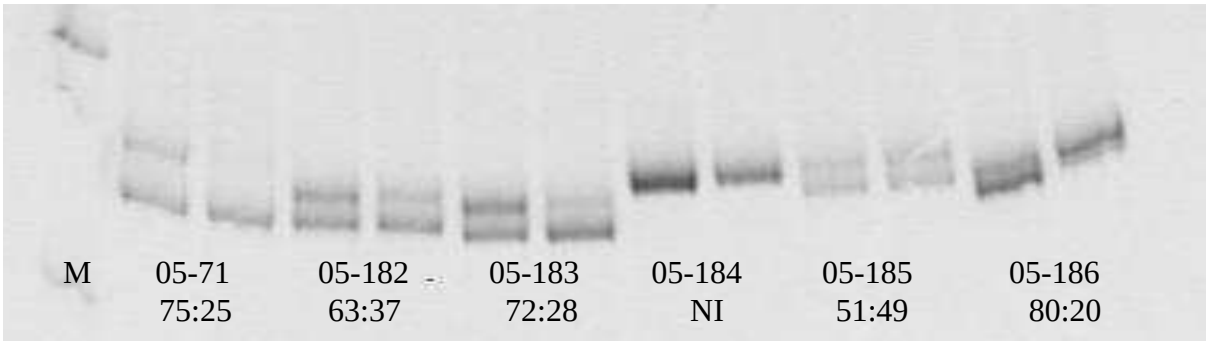
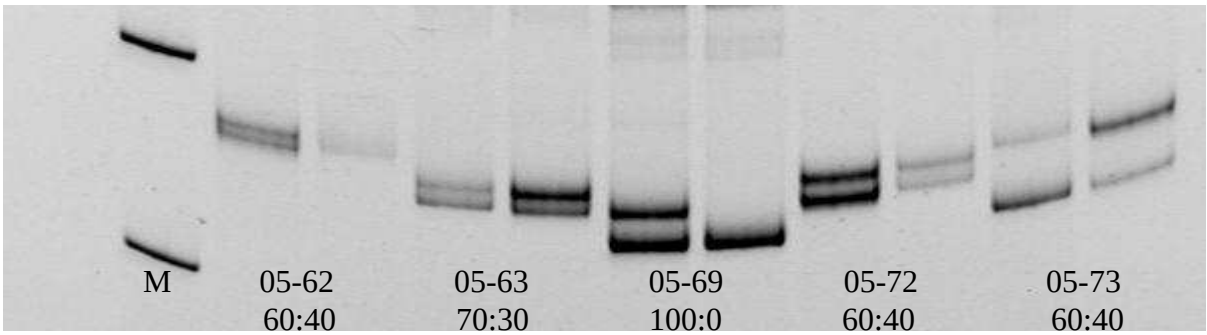
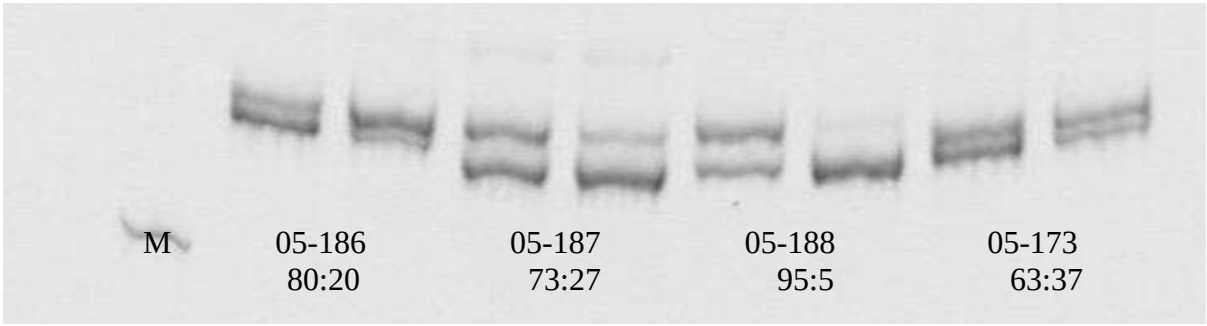


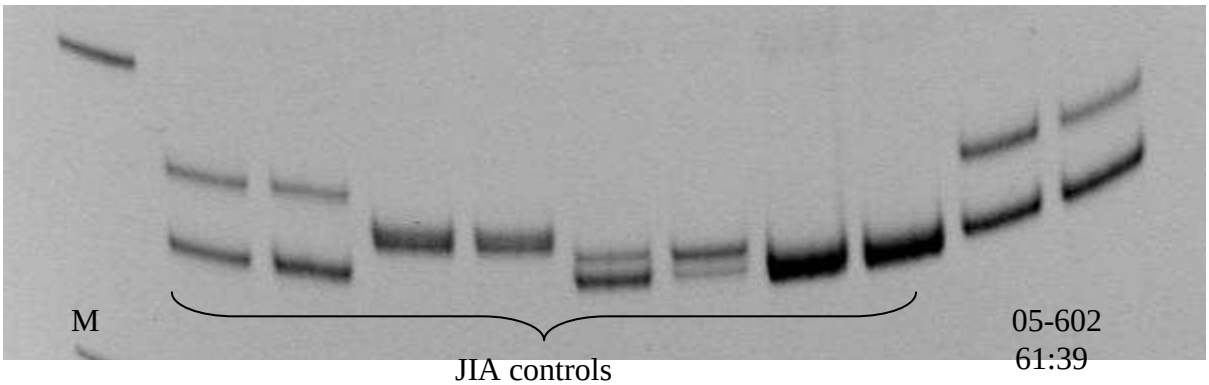
GEL6



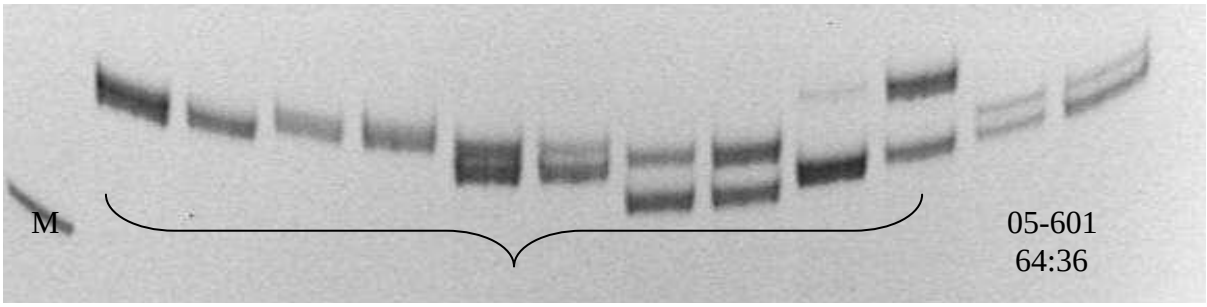
GEL7



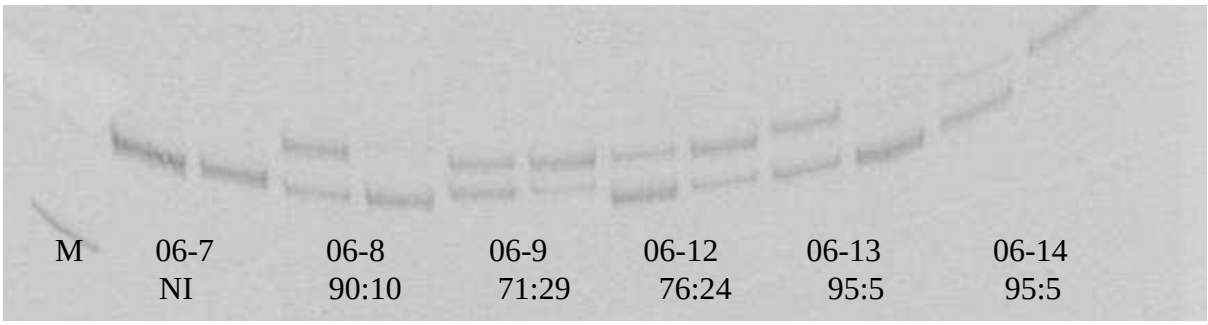
GEL8



GEL9



GEL10



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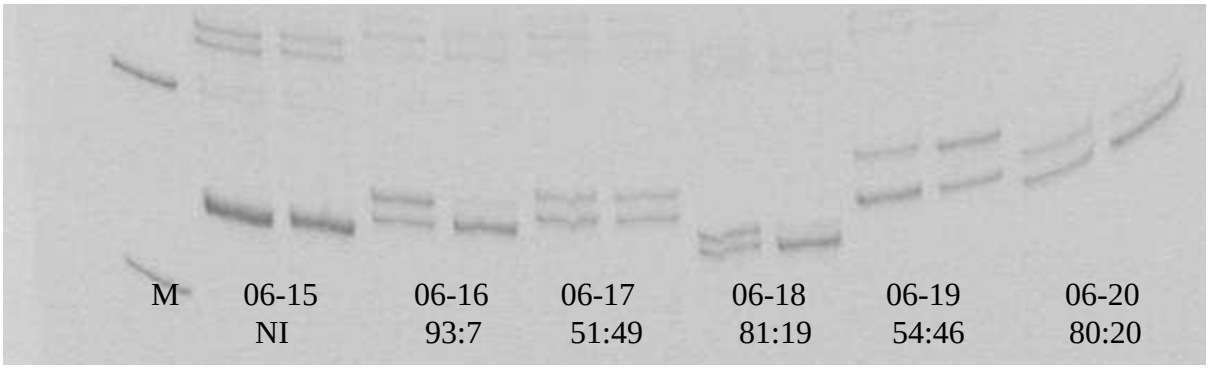

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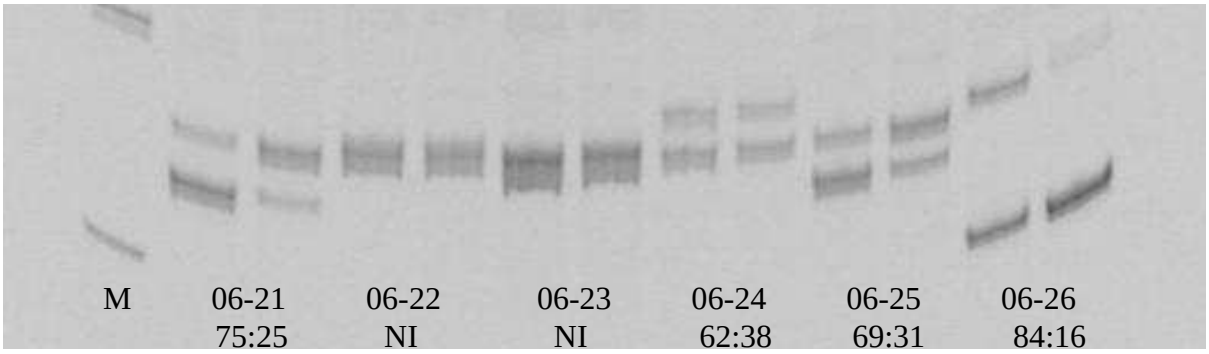

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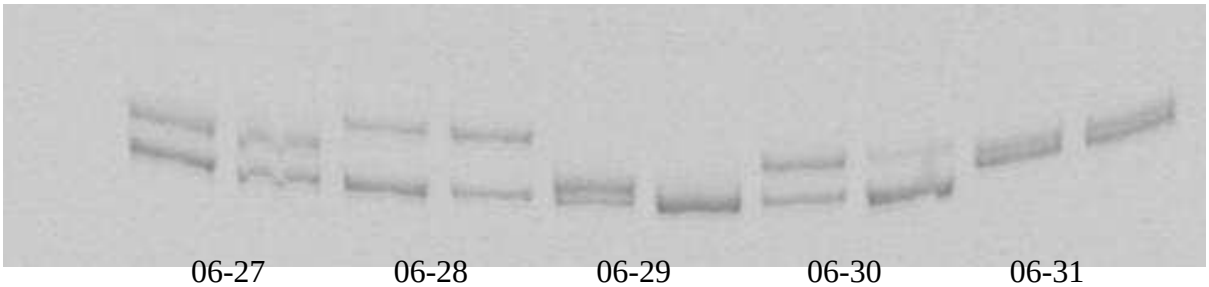

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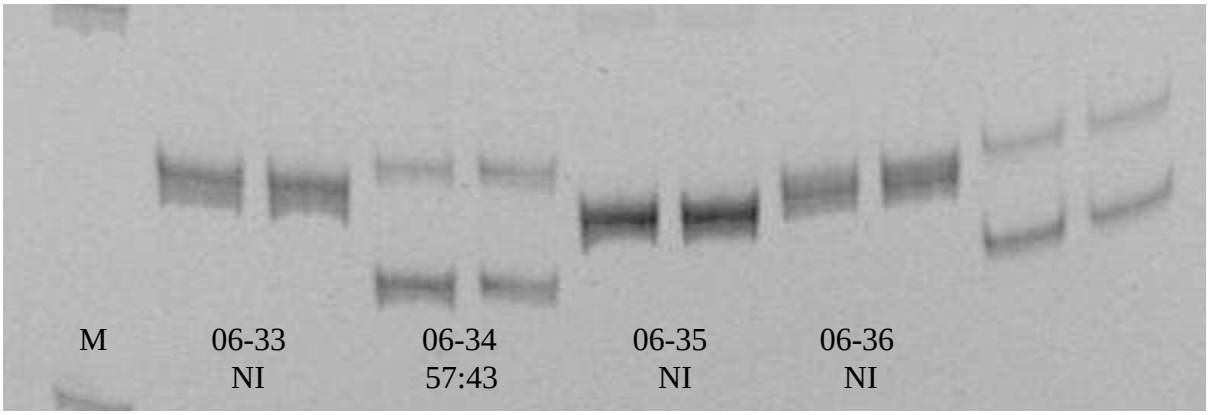
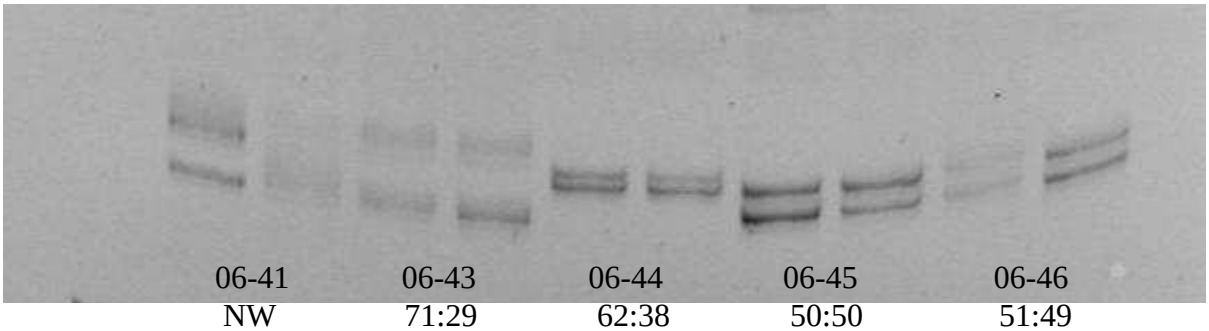
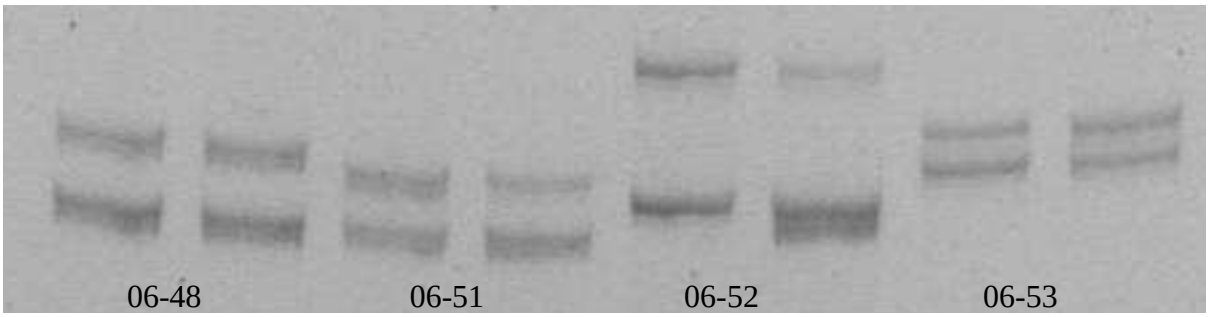


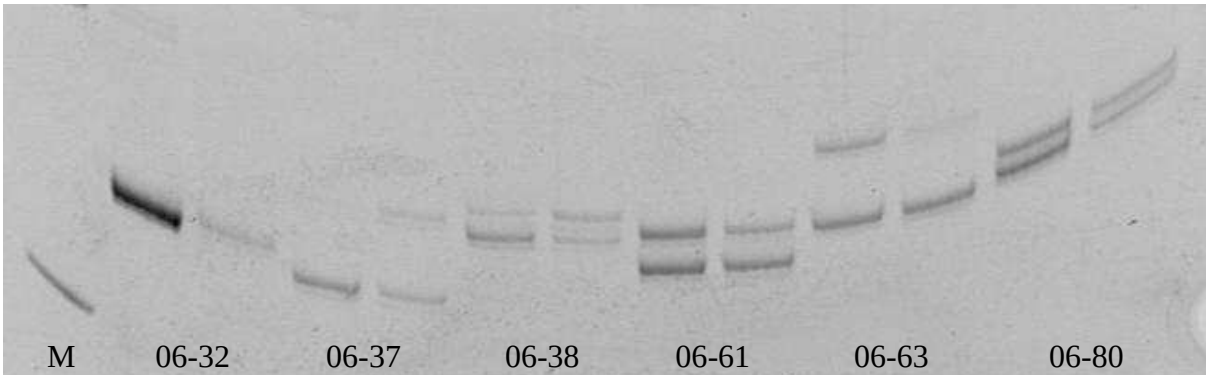
GEL15



GEL16

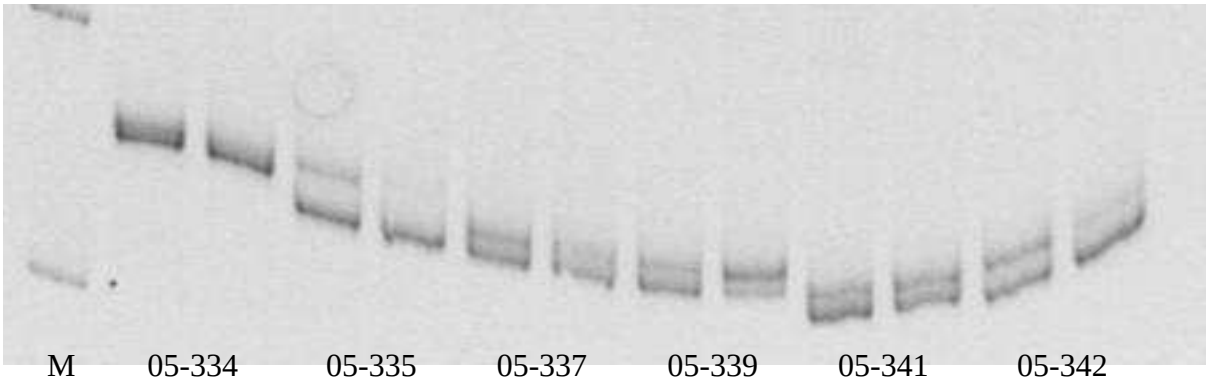


GEL17



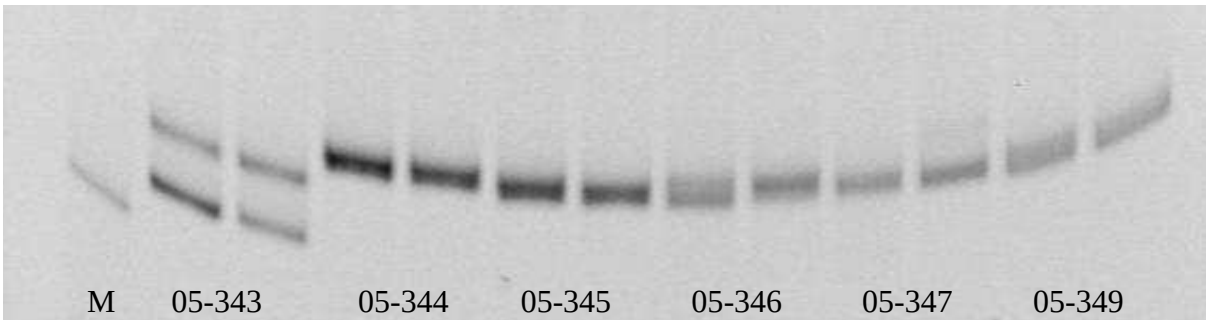
Appendix F The PAGE figures of XCI patterns of healthy controls

GEL1



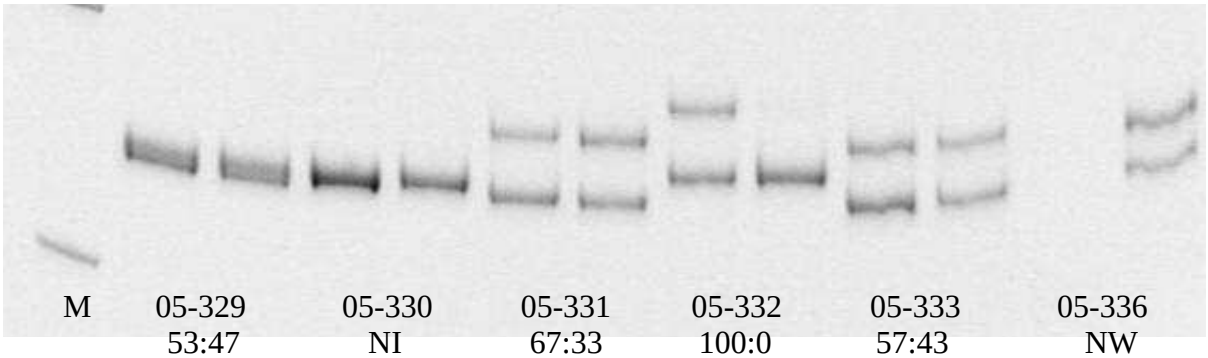
M	05-334	05-335	05-337	05-339	05-341	05-342
	NW	NW	55:45	60:40	58:42	72:28

GEL2



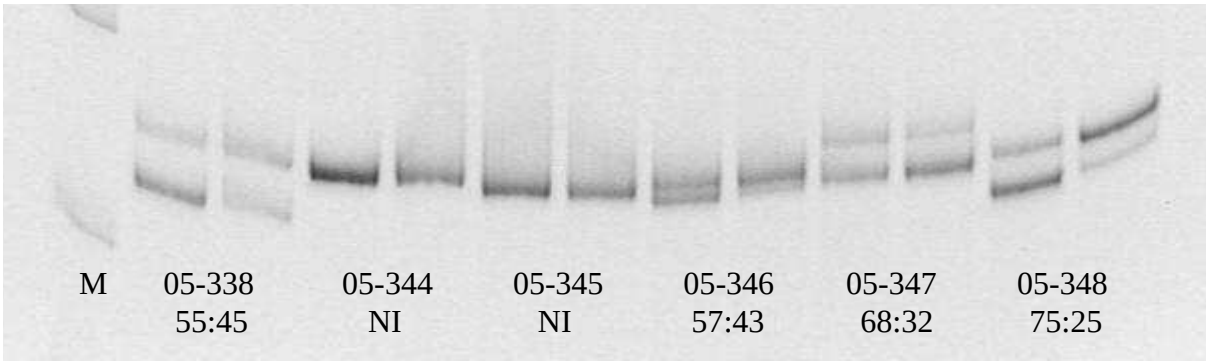
M	05-343	05-344	05-345	05-346	05-347	05-349
	50:50	NI	NI	57:43	68:32	54:46

GEL3

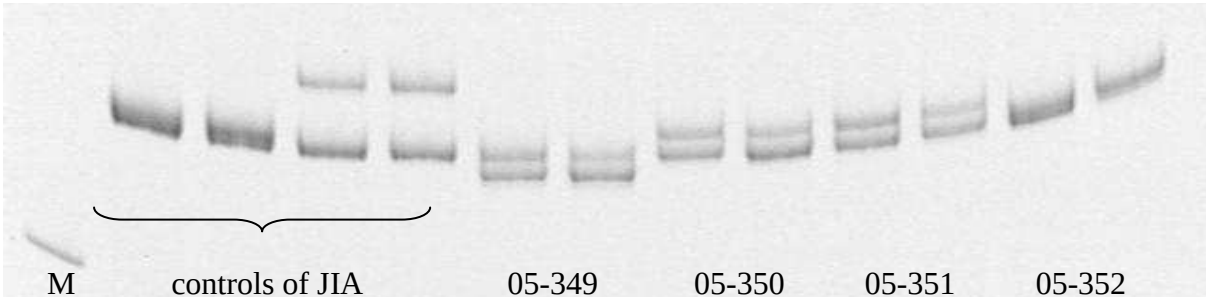


M	05-329	05-330	05-331	05-332	05-333	05-336
	53:47	NI	67:33	100:0	57:43	NW R(55:45)

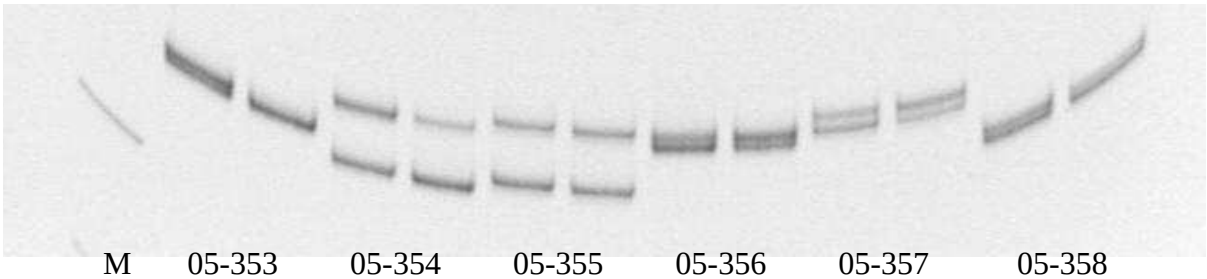
GEL4



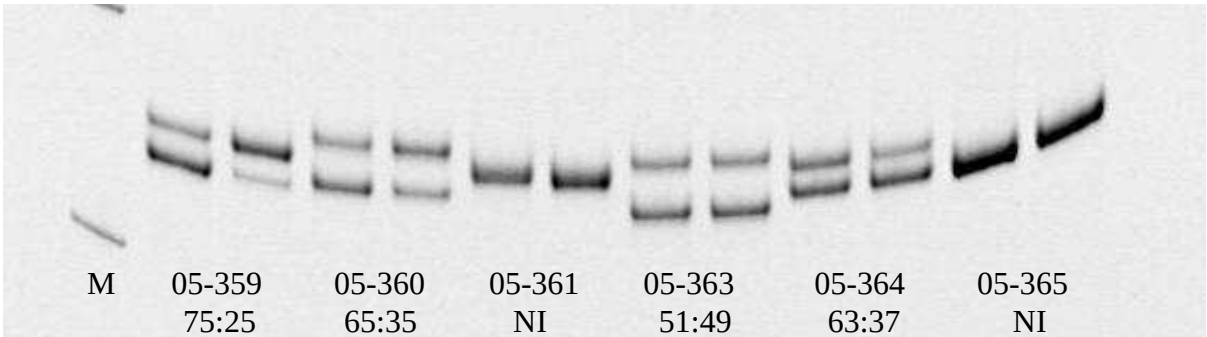
GEL5



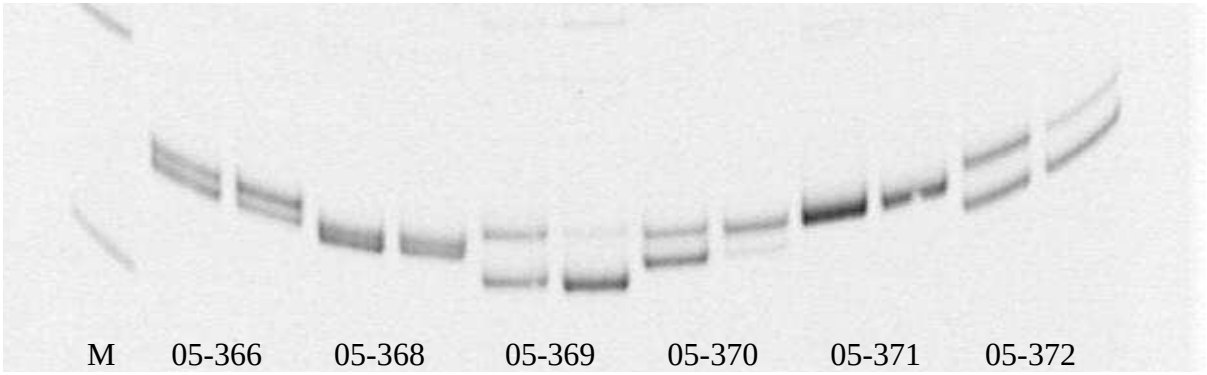
GEL6



GEL7

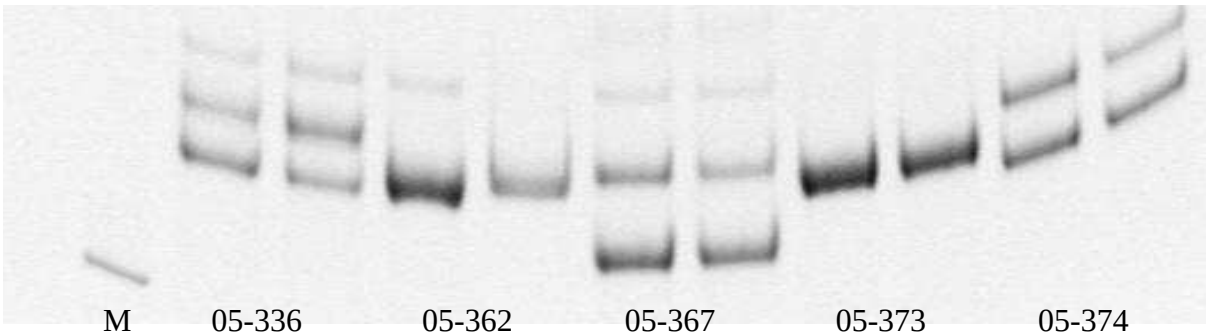


GEL8



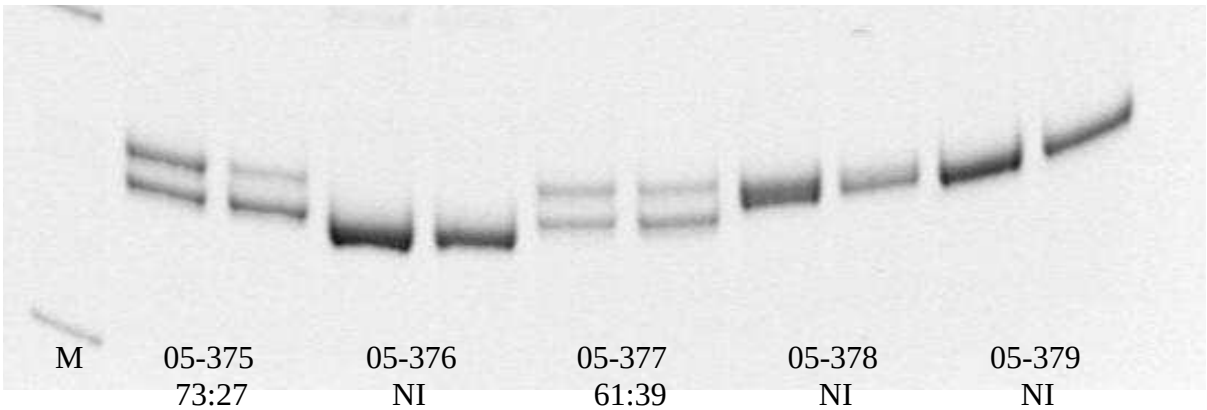
M	05-366	05-368	05-369	05-370	05-371	05-372
	64:36	55:45	85:15	85:15	NI	73:27

GEL9



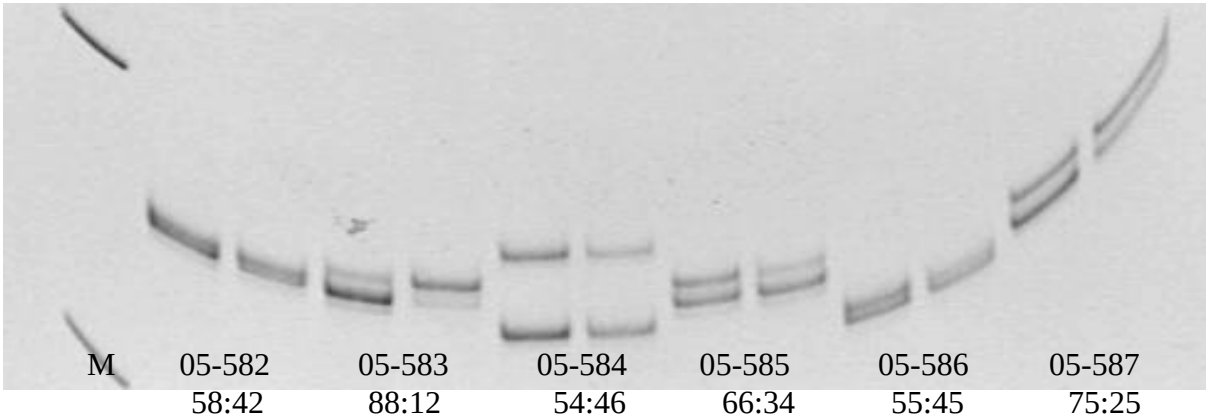
M	05-336	05-362	05-367	05-373	05-374
	55:45	NI	58:42	NI	61:39

GEL10



M	05-375	05-376	05-377	05-378	05-379
	73:27	NI	61:39	NI	NI

GEL11



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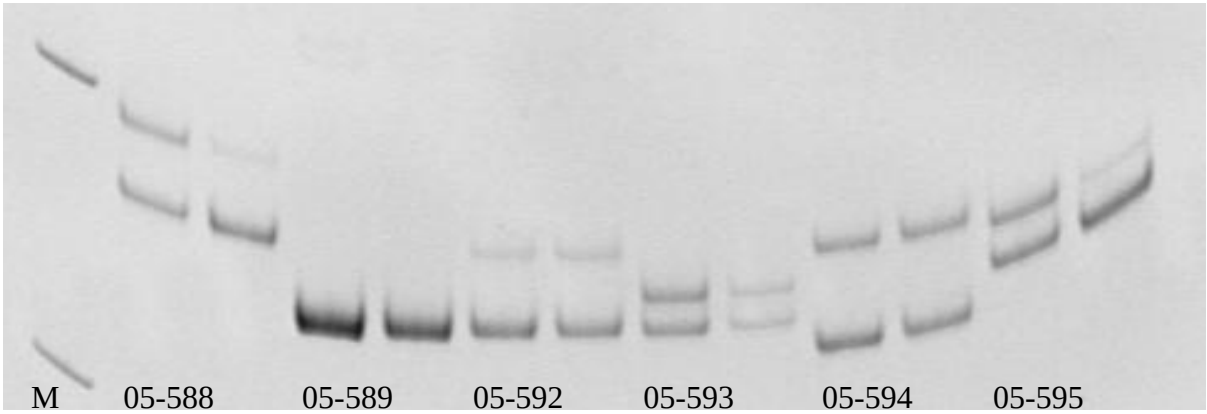
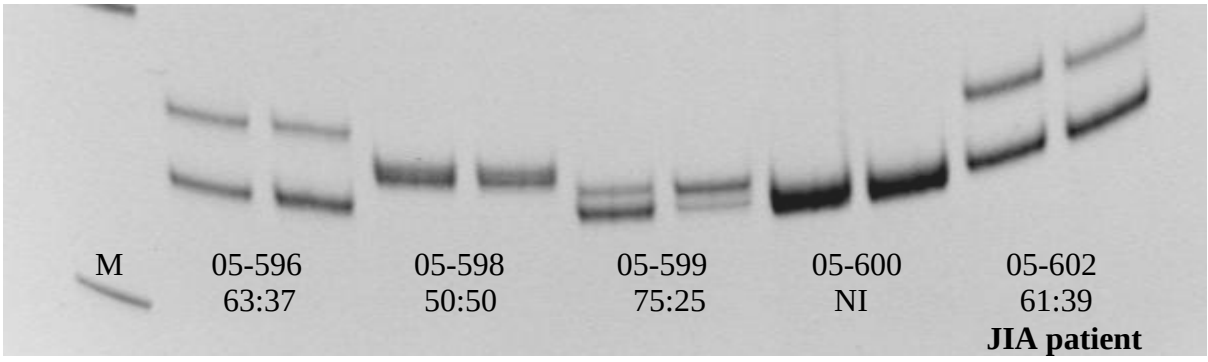
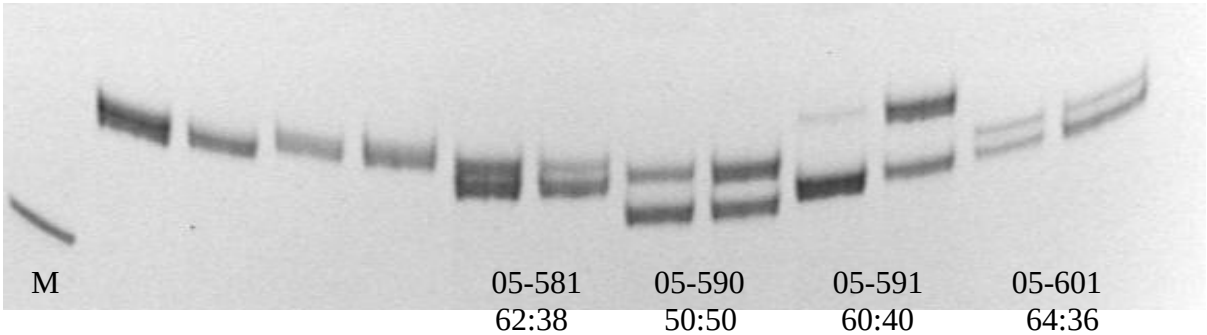


GEL13

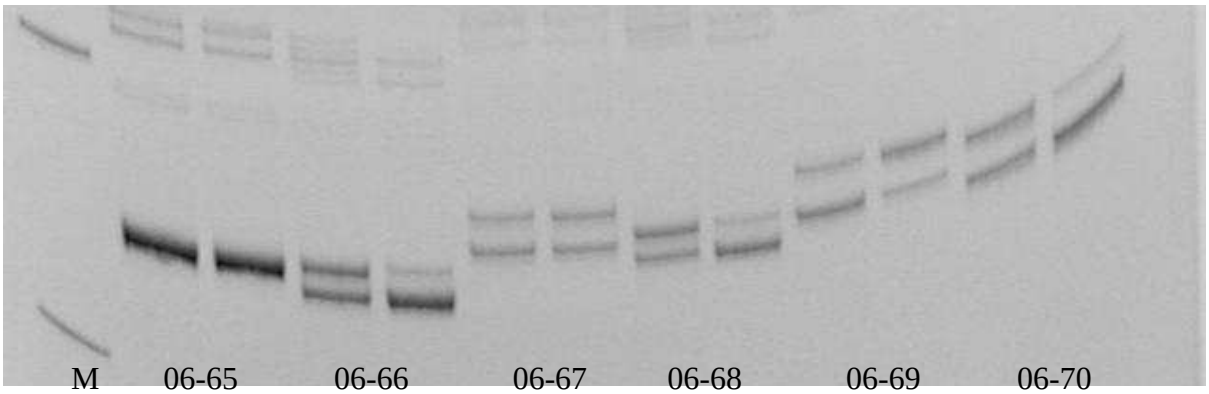


GEL14

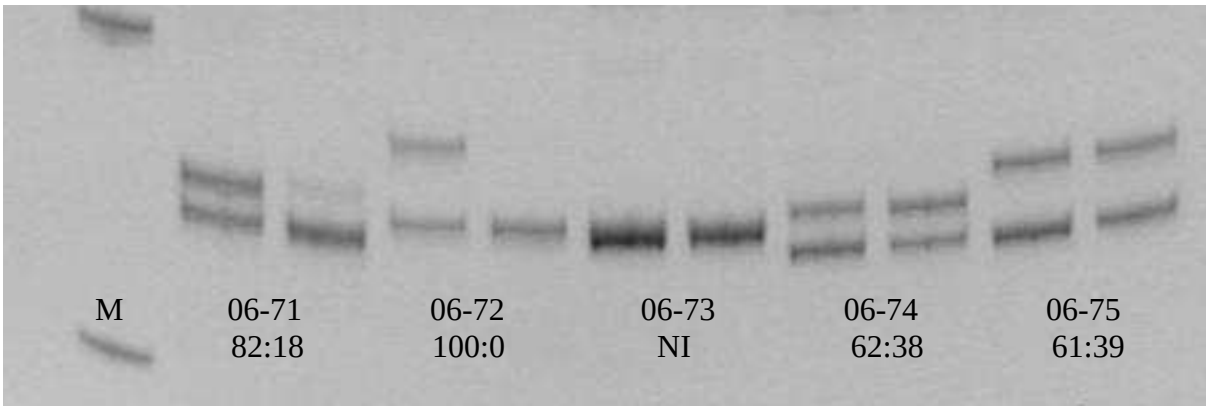


JIA patient

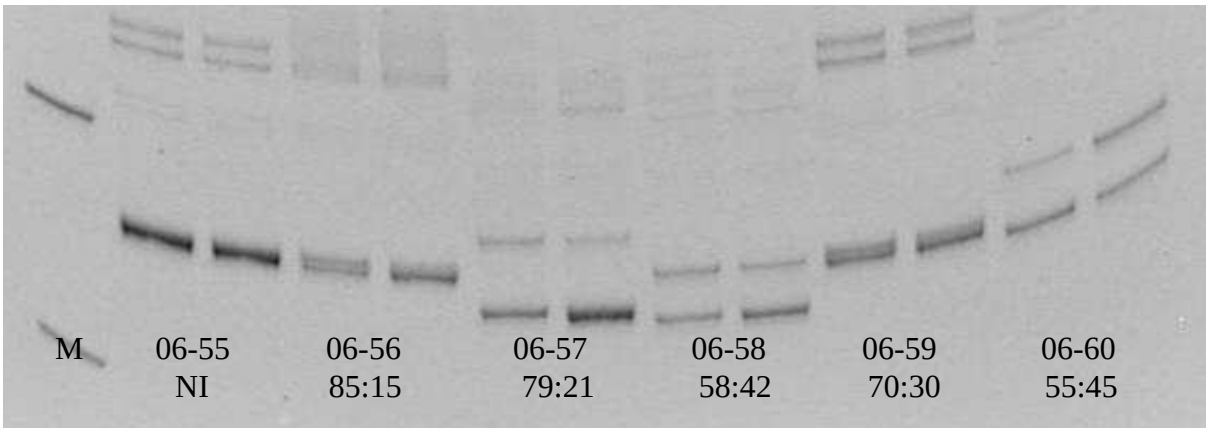
GEL15



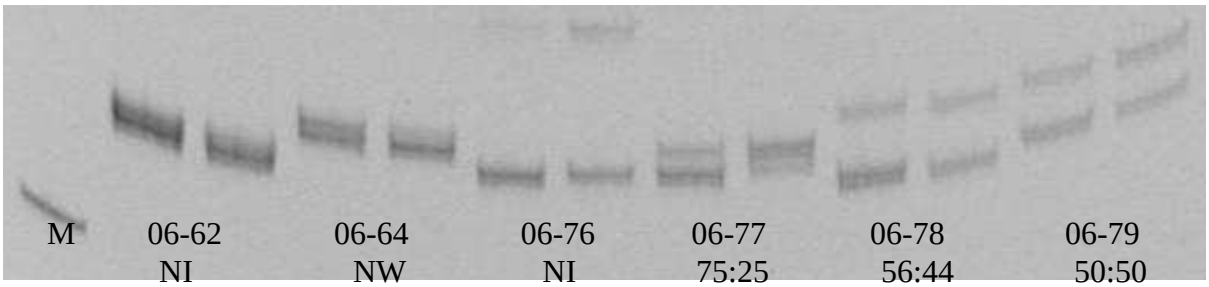
GEL16



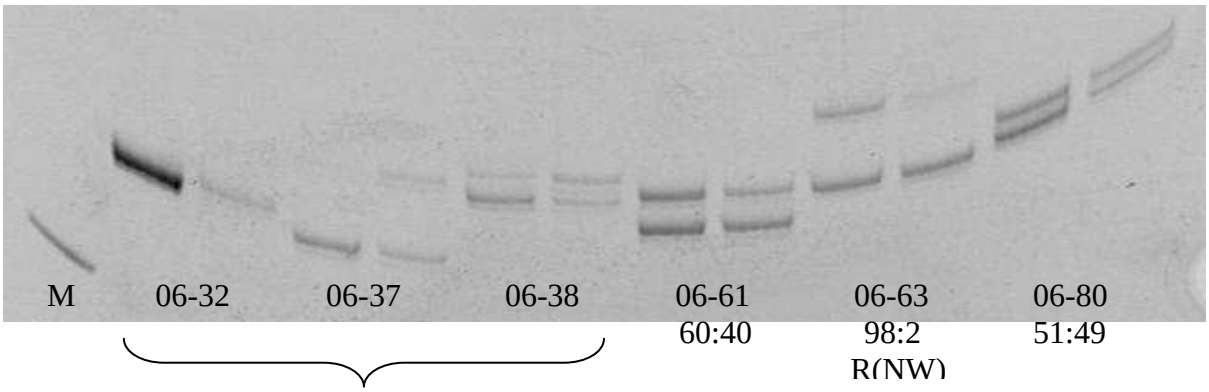
GEL17



GEL18

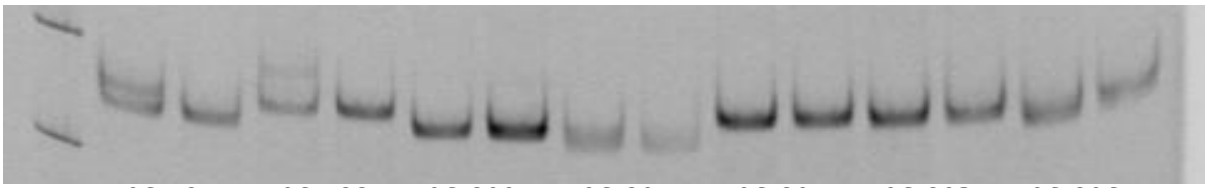


GEL19



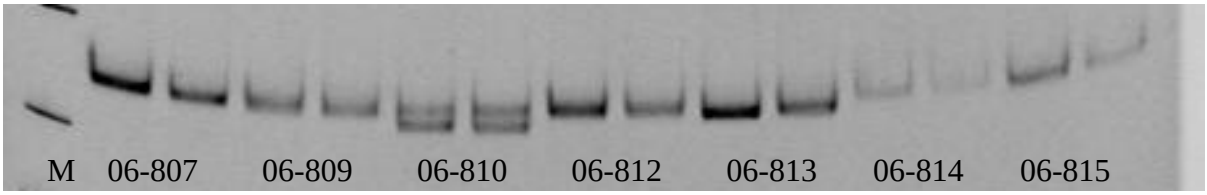
Appendix G The PAGE figures of XCI patterns of healthy newborns

GEL1



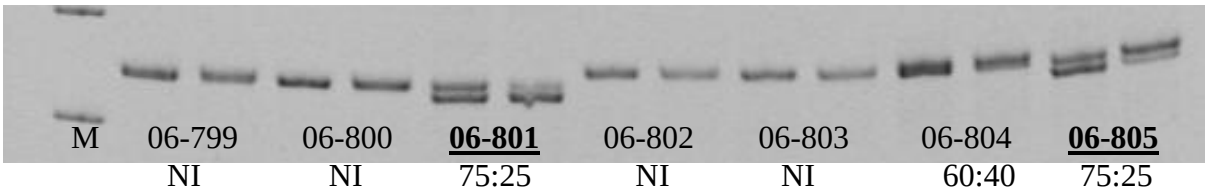
M	**06-797**	**06-798**	06-800	06-801	06-802	06-803	06-806
	100:0 R(68:32)	100:0 R(NW)	NI	NI	NI	NI	NI

GEL2



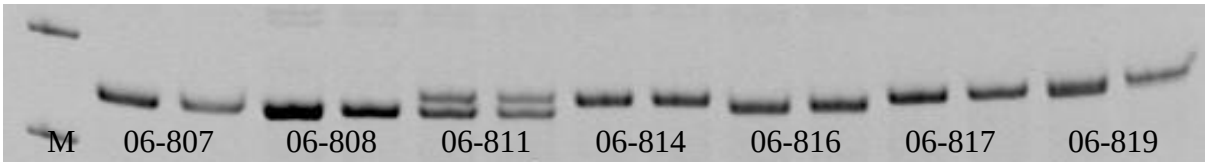
M	06-807	06-809	06-810	06-812	06-813	06-814	06-815
	NI	NI	58:42	NI	NI	NI	NI

GEL3



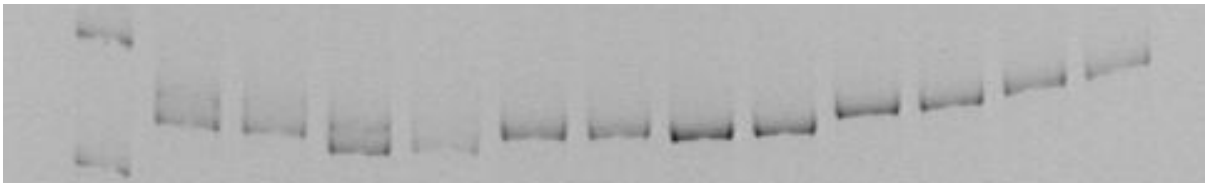
M	06-799	06-800	**06-801**	06-802	06-803	06-804	**06-805**
	NI	NI	75:25	NI	NI	60:40	75:25

GEL4



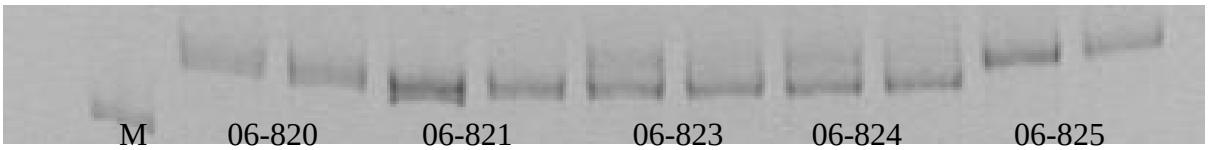
M	06-807	06-808	06-811	06-814	06-816	06-817	06-819
	NI	NI	61:39	NI	NI	NI	NI

GEL5



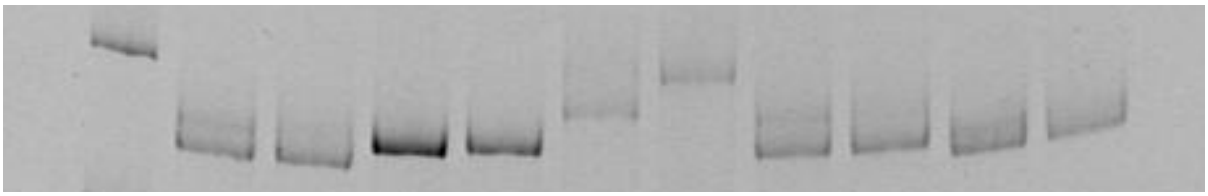
M	06-797	06-810	06-812	06-813	06-815	06-818
	68:32	58:42	NI	NI	NI	NI

GEL6



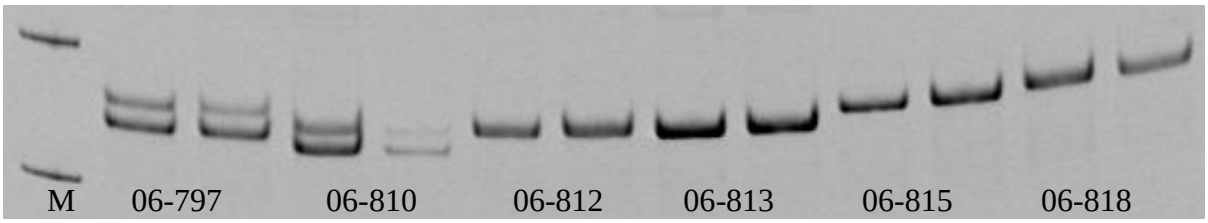
M	06-820	06-821	06-823	06-824	06-825
	61:39	NI	75:25	75:25	NI

GEL7



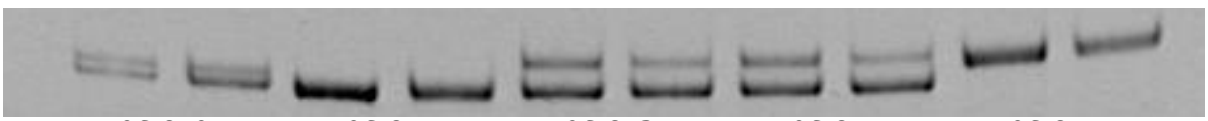
M	06-826	06-829	06-830	06-831	06-832
	60:40	NI	100:0	58:42	50:50

GEL8



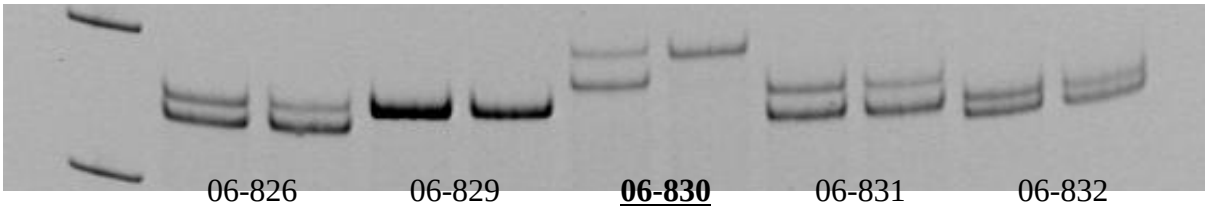
M	06-797	06-810	06-812	06-813	06-815	06-818
	68:32	58:42	NI	NI	NI	NI

GEL9



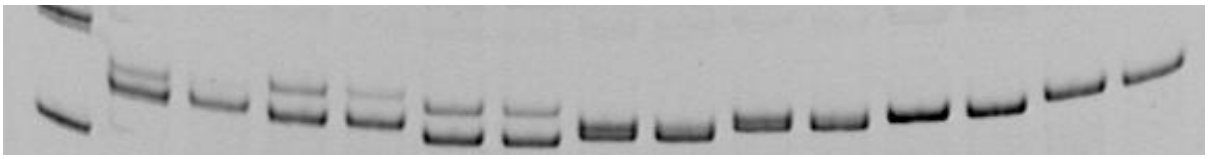
06-820	06-821	06-823	06-824	06-825
61:39	NI	75:25	75:25	NI

GEL10



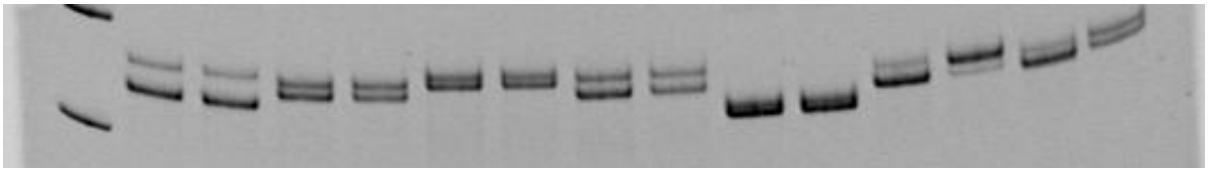
06-826	06-829	**06-830**	06-831	06-832
60:40	NI	100:0	58:42	50:50

GEL11



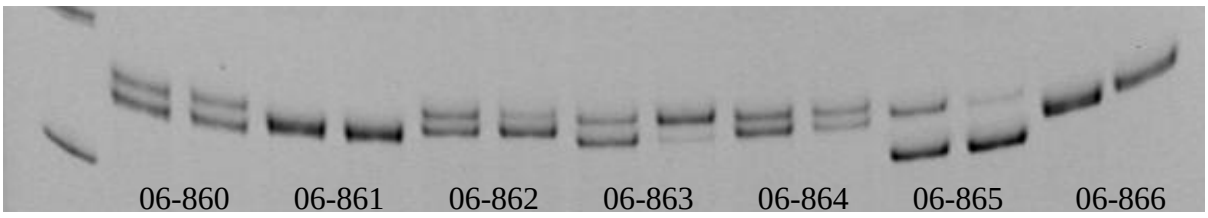
06-836	06-837	06-838	**06-839**	**06-840**	06-841	06-842
99:1	78:22	65:35	95:5	98:2	NI	NI
R(55:45)			R(NI)	R(NI)		

GEL12



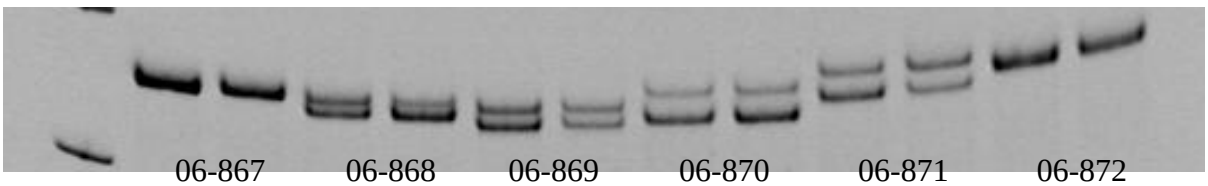
06-843	06-845	06-846	06-847	06-849	06-850	06-851
71:29	52:48	53:47	61:39	NI	78:22	66:34

GEL13



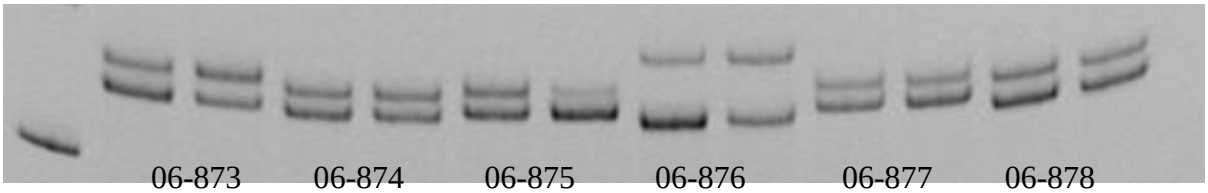
06-860	06-861	06-862	06-863	06-864	06-865	06-866
51:49	NI	66:34	82:18	57:43	80:20	NI

GEL14



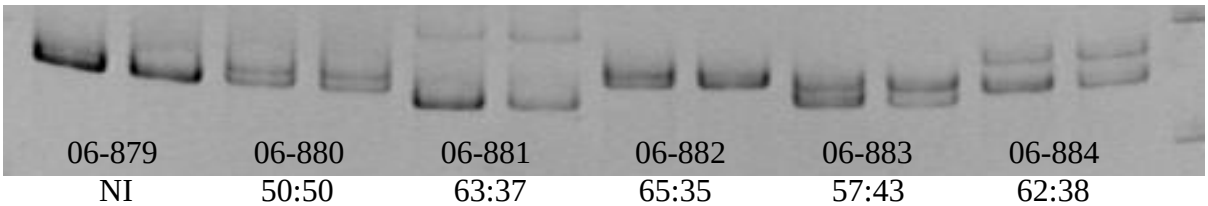
06-867	06-868	06-869	06-870	06-871	06-872
NI	77:23	58:42	67:33	55:45	NI

GEL15



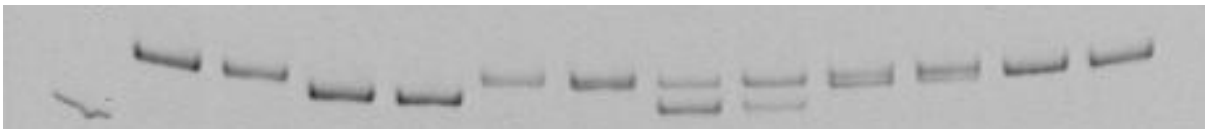
06-873	06-874	06-875	06-876	06-877	06-878
54:46	50:50	78:22	50:50	62:38	52:48

GEL16



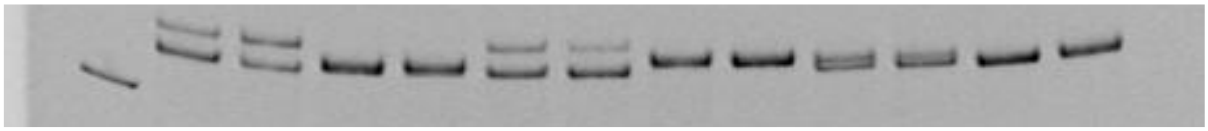
06-879	06-880	06-881	06-882	06-883	06-884
NI	50:50	63:37	65:35	57:43	62:38

GEL17



06-885	06-886	06-887	06-888	06-889	06-890
NI	NI	NI	54:46	68:32	NI

GEL18

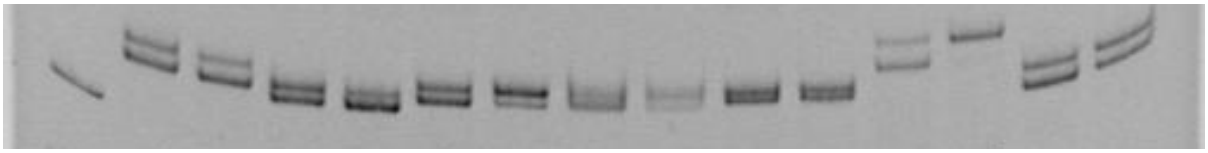


06-891	06-892	06-893	06-894	06-896	06-897
59:41	NI	72:38	NI	77:23	NI

GEL19

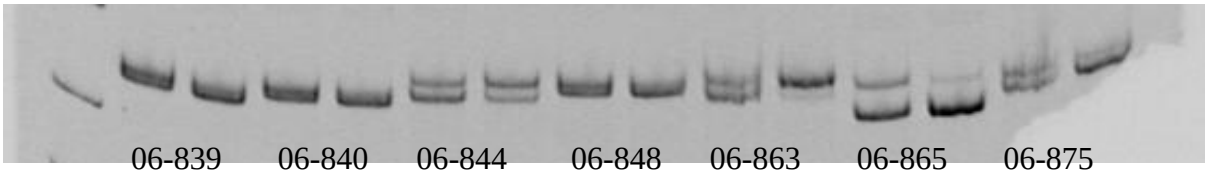
06-899	06-900	06-822	06-827	06-828
NI	65:35	50:50	64:36	NI

GEL20 (repeat for checking)



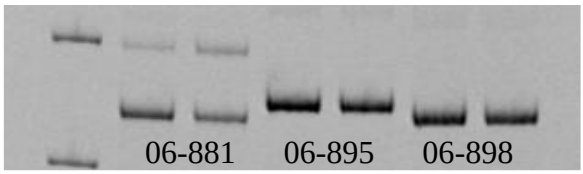
06-797	06-801	06-805	06-806	06-822	06-830	06-836
68:32	75:25	75:25	NW	50:50	100:0	55:45

GEL21 (repeat for checking)



06-839	06-840	06-844	06-848	06-863	06-865	06-875
NI	NI	70:30	99:1	82:18	80:20	78:22

GEL22 (repeat for checking)



06-881	06-895	06-898
63:37	NI	NI