

Genetics and Biomarkers of Osteoarthritis and Joint Hypermobility

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Pathology in the Graduate School
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ABSTRACT

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Osteoarthritis (OA) is the most common joint disorder causing chronic disability in the world population. By the year 2030, an estimated one fifth of this population will be affected by OA. Although OA is regarded as a multi-factorial disorder with both environmental and genetic components, the exact pathogenesis remains unknown.

In this study, we hypothesize that biomarkers associated with OA can be used as quantitative traits of OA, and provide enough power to identify new genes or replicate known gene associations for OA. We established an extensive family called the CARRIAGE (CARolinas Region Interaction of Aging, Genes and Environment) family. Then, we measured and analyzed seven OA-related biomarkers (HA, COMP, PIIANP, CPII, C₂C, hs-CRP and GSP) in this extensive family to evaluate their association with OA clinical phenotypes. These findings suggest that OA biomarkers can reflect hand OA in this large multigenerational family. Therefore, we performed nonparametric variance components analysis to evaluate heritability for quantitative traits for those biomarkers. Finally, based upon OA biomarkers with high heritability, we performed a genome-wide linkage scan. Our results provide the first evidence of genetic susceptibility loci identified by OA-related biomarkers, indicating several genetic loci potentially contributing to the genetic diversity of OA.

Meanwhile, we identified joint hypermobility as a factor which reduces OA risk and has an inverse association with serum COMP levels in this family. The relationship between lower serum COMP and OA have been further validated in another Caucasian GOGO (Genetics of Generalized Osteoarthritis) population. Therefore, we further hypothesize that joint hypermobility, having the characteristic of a decreased OA risk, can serve as a quantitative trait for identifying protective loci for OA. Then, we performed nonparametric variance components analysis to evaluate the heritability of joint hypermobility. The result also shows joint hypermobility has substantial heritable components in this family. Lastly, based on the same genome-wide linkage scan, we identify genetic susceptibility loci for joint hypermobility.

In conclusion, our work provides the first linkage study to identify genetic loci associated with OA using biological markers. Furthermore, we have also shown genetic susceptibility loci for joint hypermobility, possibly implying protective loci for OA.

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LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
ADAMTS	ADAM metalloproteinase with thrombospondin type 1
ADAMTS3	ADAM metalloproteinase with thrombospondin type 1 motif, 3
AGC1	aggrecan 1
AGE	advanced glycation end product
ANGPT2	angiopoietin 2
ANK1	ankyrin 1
ASPN	asporin
BJHS	benign joint hypermobility syndrome
BMP	bone morphogenetic proteins
BMP2	bone morphogenetic protein 2
CALM1	calmodulin 1
CARRIAGE	CARolinas Region Interaction of Aging, Genes and Environment
CD36	platelet glycoprotein 4
CMC	carpometacarpal
COL10A1	collagen, type X, alpha 1

COL11A2	collagen, type XI, alpha 2
COL22A1	collagen, type XXII, alpha 1
COMP	cartilage oligomeric matrix protein
CTSL	cathepsin L
CTX-1	C-terminal cross-linked telopeptide of type I collagen
DEFB4	beta-defensin 4
DIO2	type II deiodinase iodothyronine
DIP	distal interphalangeal
DVWA	von Willebrand factor A domains
ECM	extracellular matrices
EDG2	endothelial differentiation, lysophosphatidic acid (LPA) GPCR, 2
ESR2	estrogen receptor 2
FLRT2	fibronectin leucine-rich transmembrane protein 2.
FRZB	frizzled-related protein
GARP	Genetics of osteoARthritis and Progression
GDF5	growth differentiation factor 5
GEE	generalized Estimating Equations
GOA	generalized OA
GOGO	Genetics of Generalized OA

GSP	glycated serum protein
GWAS	genome-wide association study
HA	hyaluronic acid
HbA1c	hemoglobin A1c
HFE	hemochromatosis
IBD	Identity-by-descent
IGF	insulin-like growth factor
IGFBP7	insulin-like growth factor binding protein 7
IL17A	interleukin 17A
IL17F	interleukin 17F
IL4R	interleukin 4 receptor
JSN	joint space narrowing
LOD	logarithm of odds
LRCH1	leucine-rich repeats and calponin homology domain-containing
MED	multiple epiphyseal dysplasia
MMP	matrix metalloproteinase;
NTX-1	N-terminal cross-linked telopeptide of type I collagen
OA	osteoarthritis
OGN	osteoglycin
OI	osteogenesis imperfect

OST	osteophyte
PIP	proximal interphalangeal
PLA2G4A	phospholipase A2, group IVA
PLAG1	pleomorphic adenoma gene 1
PLAT	plasminogen activator, tissue
PSACH	pseudoachondroplasia
PTGS2	prostaglandin-endoperoxide synthase 2
QTL	quantitative trait locus
RK	residual kurtosis
SLC26A2	solute carrier family 26
SNP	single nucleotide polymorphisms
SOD3	superoxide dismutase 3, extracellular
SOLAR	Sequential Oligogenic Linkage Analysis Routines
TGFB1	transforming growth factor, beta 1
TNFAIP6	tumor necrosis factor, alpha-induced protein 6
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b
U-DIP	unaffected distal interphalangeal
U-JSN	unaffected joint-space narrowing
U-OST	unaffected osteophyte
WISP1	wnt-1-induced secreted protein 1

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CHAPTER 1

Background and Literature Review

1.1 Overview of Osteoarthritis (OA)

OA is the most common type of arthritis leading to the morbidity and disability of millions of people worldwide (Brooks 2002). It is estimated that 60% of men and 70% of women, over the age of 65, are affected by OA. The WHO characterized OA and Alzheimer's disease as "high burden diseases with no curative treatments"(Kaplan W 2004). Furthermore, there is currently no effective treatment available for halting OA progression. Kofi Annan, the previous Secretary-General of the United Nations signed the declaration to commence the Bone and Joint Decade (2000-2010) for treatment and prevention of musculo-skeletal disorders. In the United States alone, the prevalence of OA has nearly reached 27 million, which has increased by around six million since 1995 (Lawrence, Helmick et al. 1998).

OA has been regarded as a disease which is strongly correlated with age. It has been shown to have increased ten folds in prevalence from age 30 to age 65 (Felson, Lawrence et al. 2000). However, development of OA is also associated with genetics, obesity, nutritional factors, performance of repetitive, previous cartilage and ligament injury (Hunter and Felson 2006).

OA has been considered to be the result of cartilage degeneration due to aging. However, the lack of OA in many elderly people implies that aging is not a universal feature of OA. Recent studies also indicated that OA is obviously not only a cartilage disorder, but rather a disease of the entire joint, including cartilage, bone, synovium, meniscus, tendon, ligaments, and peri-articular muscles (Quasnicka, Anderson-MacKenzie et al. 2006; Mansell, Collins et al. 2007). Although the precise etiology of OA remains unknown, current knowledge believe that OA may not be a single disease entity, but a group of overlapping distinct diseases, which may have a similar initiation point, even though from different etiologies (Figure 1.1).

1.1.1 Normal Structure of Joint and Articular Cartilage

Joints are highly specialized organs which tolerate repetitive and frictionless movements. These assets are provided by the articular cartilage and extracellular matrix. Under normal physiological conditions, joints are capable of withstanding high mechanical loading. Joints are complicated composites with different types of connective tissue which extend over articular cartilage. These are composed of ligaments, synovium, subchondral bone, and joint capsules, providing different and reciprocal functioning of the joint (Figure 1.2).

Normal articular cartilage is composed of a substantial hydrated extracellular matrix and chondrocytes. The extracellular matrix is composed of a collagen network (mostly type II with lesser amounts of type IX, XI and other collagens) and provides tensile strength to maintain the retention of proteoglycans.

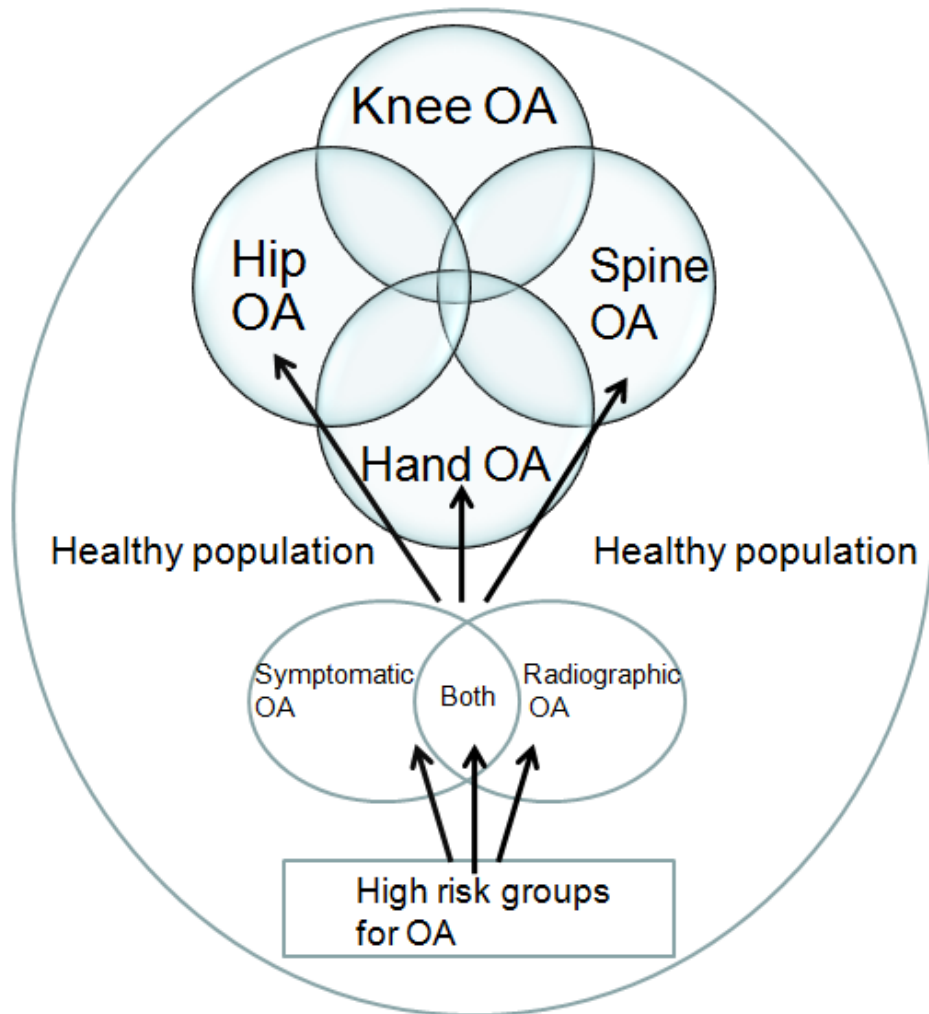


Figure 1.1: Is OA a single disease or a group of overlapping distinct diseases?

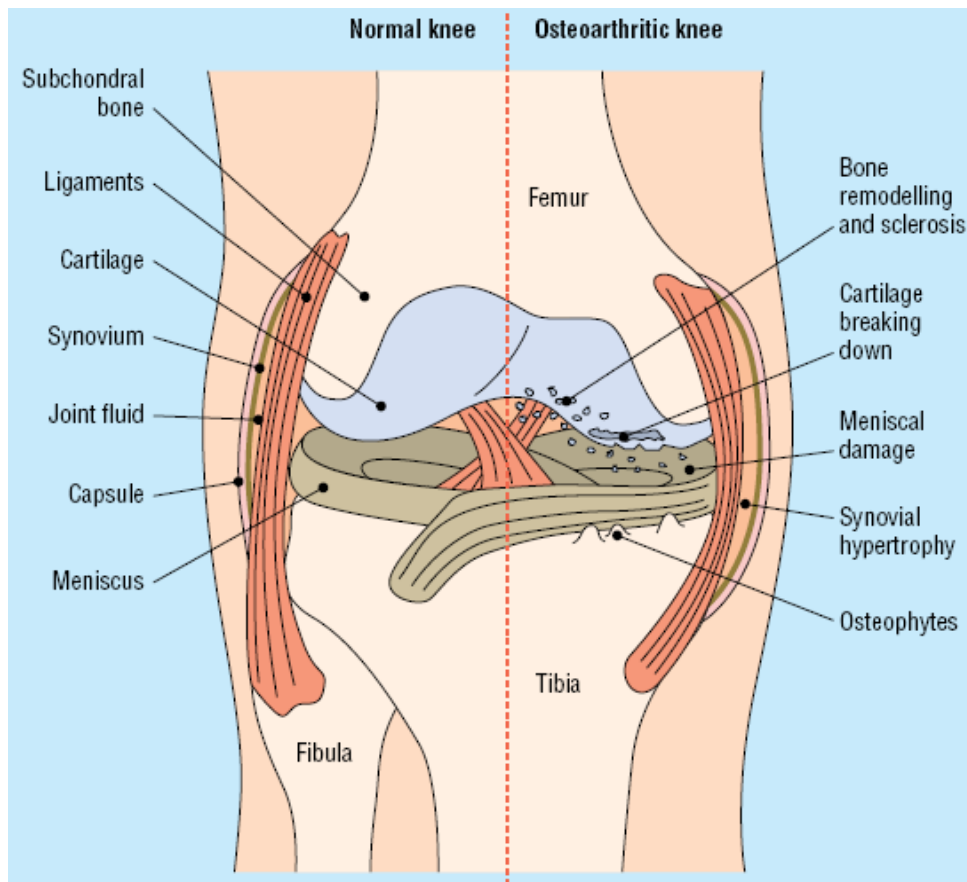


Figure 1.2: Normal structure of knee (Lt) and pathogenic changes observed in OA joints (Rt). Adapted from Hunter, D. J. and D. T. Felson (2006). "Osteoarthritis." *BMJ* 332(7542): 639-42.

The proteoglycans (mostly aggrecan) are aggregated with hyaluronic acid (HA). Other large components, including non-collagenous proteins, contribute to the unique properties of the matrix (Roach, Aigner et al. 2007). Non-collagenous proteins include cartilage oligomeric matrix protein (COMP), biglycan, tenascins, matrilins, and fibronectins, etc. Chondrocytes comprise 2-5% of the total volume and are fairly inactive metabolically, owing to the absence of a vascular supply.

1.1.2 Classification and Etiology of OA

OA can exist in two main types: primary (idiopathic) OA and secondary OA. Primary OA is the common late-onset form without an obvious predisposing factor such as joint injury or developmental abnormalities. Secondary OA usually arises from trauma, and/or a congenital or a developmental abnormality (table 1.1). Because primary OA has the most significant impacts in a population, primary OA will be focus in the following chapters. OA also can be classified as localized or as generalized forms of OA. Localized OA refers to the OA confined to one joint; generalized OA (GOA) refers to the involvement in the disease of at least three joints or a group of joints. OA is a multifactorial disease involving endogenous and exogenous factors (Felson, Lawrence et al. 2000). The endogenous factors include sex, age, race and genes; the exogenous environmental factors consist of joint load, injury, estrogen replacement therapy, vitamins C and D intake. In general, OA has a higher prevalence rate in females;

however, men have a higher prevalence rate than females before the age of 50. Ethnic difference is also associated with OA susceptibility. For example, hip OA is less prevalent in Pima and Blackfoot Native Americans, and Asians (Felson and Zhang 1998). Recent several studies also have shown that genetic factors play roles in OA susceptibility and progression. Having these risk factors along and/or together with exogenous factors may result in the initiation of the disease (Jordan, Kraus et al. 2004; Peach, Carr et al. 2005) (Figure 1.3).

Table 1.1 Short summary of classification of OA

Classification of Osteoarthritis (OA)	
Primary (idiopathic)	Secondary
Peripheral joints	Trauma
single vs. multiple joints	Acute injury
Interphalangeal joints	Chronic
Distal Interphalangeal OA	Occupation-related
Proximal Interphalangeal OA	Sports-related
Other small joints	Obesity
First carpometacarpal OA	Familial OA
First metacarpophalangeal OA	Skeletal dysplasias
Large joints	Pseudoachondroplasia (PSACH)
Knee OA	Multiple epiphyseal dysplasia (MED)
Hip OA	

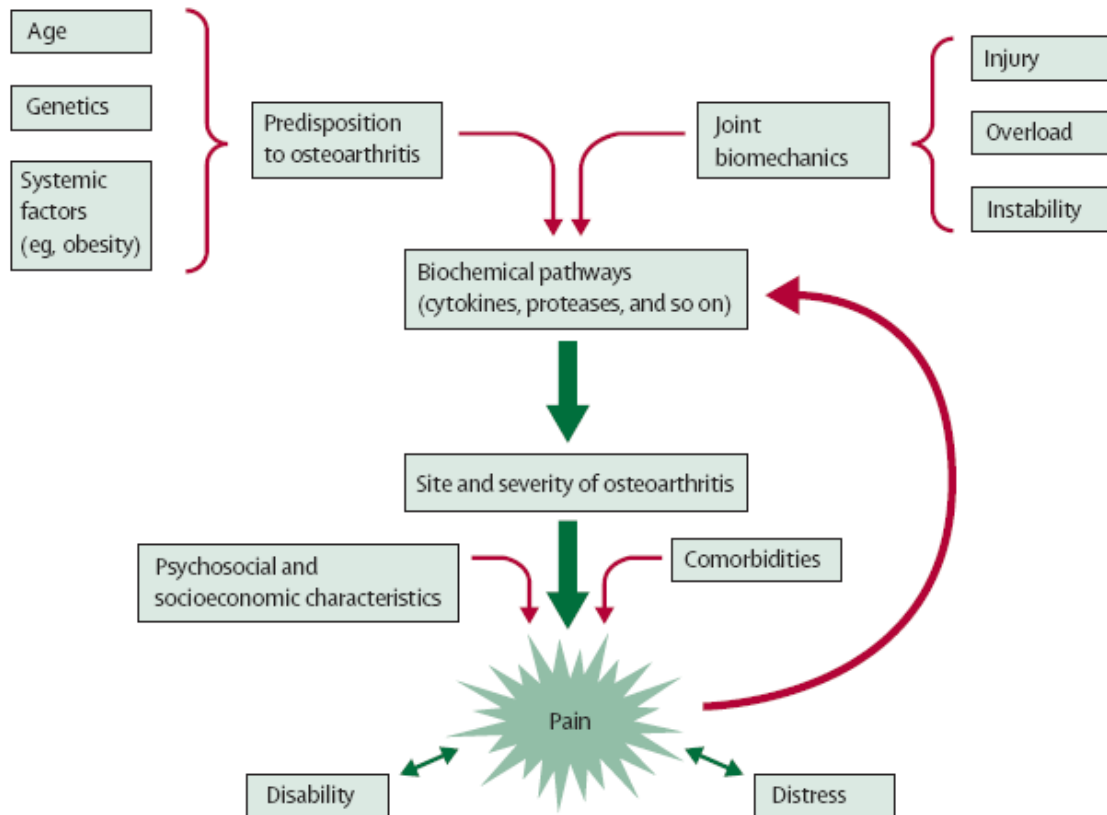


Figure 1.3: Schematic representation of relations between environmental and endogenous risk factors for joint. Adapted from Dieppe, P. A. and L. S. Lohmander (2005). "Pathogenesis and management of pain in osteoarthritis." *Lancet* 365(9463): 965-73.

1.1.3 Clinical Manifestations, Current Diagnosis of OA

OA can occur in any joint of the body, but the most commonly affected sites are the joints of the hands, knees, hips and spine. When OA develops into being clinically obvious, the symptoms include joint pain, stiffness, tenderness, crepitus, swelling, limitation of movement and finally instability and deformity of the affected joint. Currently, the diagnosis of OA is based on clinical criteria and radiographic changes. The clinical criteria include ACR (American College of Rheumatology) (Altman, Asch et al. 1986; Altman, Fries et al. 1987; Altman, Alarcon et al. 1990) and GOGO (Genetics of Generalized OA) criteria (Kraus, Jordan et al. 2007). Since it is not satisfied sensitivity and specificity based on clinical evaluation only, radiography has been regarded as the gold standard for diagnosis of OA. The radiographic changes are evaluated by joint space narrowing (JSN), which is an indirect measurement of cartilage loss, the formation of osteophytes, subchondral bone sclerosis, and subchondral cysts.

Furthermore, many OA affected individuals defined by radiographic evidence do not suffer from OA symptoms. On the other hand, many OA affected people in the early stages do have OA symptoms. For these reasons, sometimes the classification of OA using radiographic versus symptomatic OA is preferred (Creamer and Hochberg 1997) (Figure 1.1).

1.1.1.4 Pathogenesis of OA

The destruction of articular cartilage and impairment of its biomechanical function are mainly due to the loss of interterritorial cartilage matrix. These result from an imbalance between cartilage degradation and the synthesis of matrix components on the molecular level despite amendable effort by the chondrocytes (Figure 1.4). The earliest pathological changes are chondrocyte proliferation and increasing synthetic activity, followed by increased cartilage-degrading proteinases and matrix proteins. At a later stage, the loss of proteoglycans and the initiation of type II collagen degradation gradually develop in the surface region of the articular cartilage. Fibrillation is noted along the articular surface, followed by osteophyte formation and subchondral bone sclerosis. Although the etiopathogenetical initiating factors are different, the pathological progression follows a similar outcome, indicating that there may have a common molecular pathway for OA progression (Li, Xu et al. 2007) (Figure 1.5).

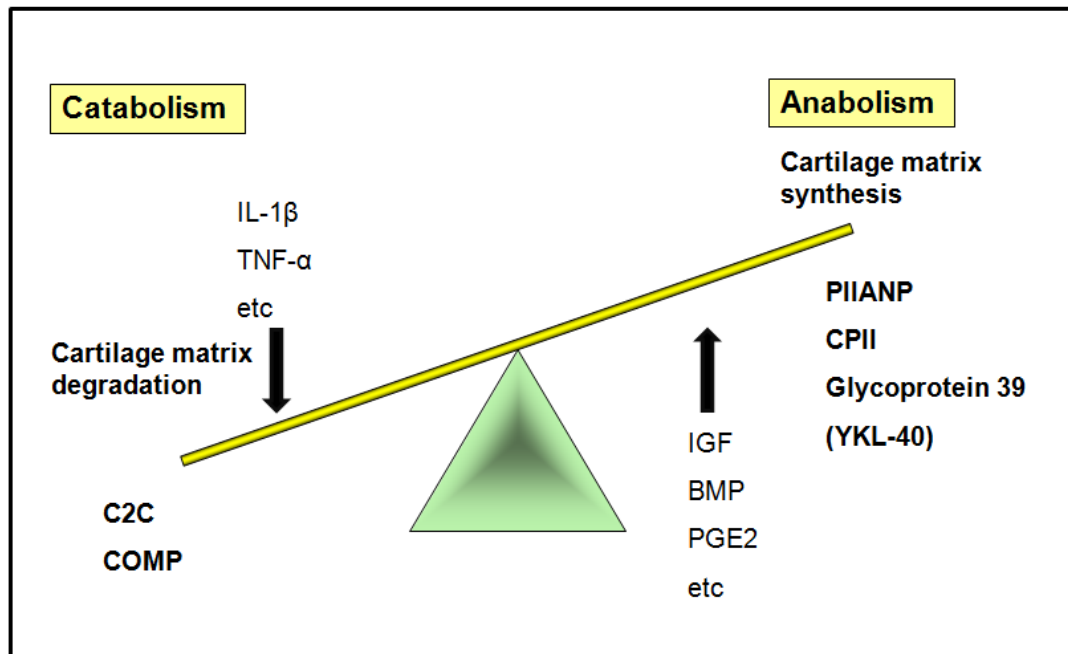


Figure 1.4: Osteoarthritis results from imbalance of cartilage matrix turnover by chondrocytes. IL-1 β = interleukin-1 β ; TNF- α = tumor necrosis factor α ; C₂C = neoepitope from cleavage of CII; COMP = cartilage oligomeric matrix protein; IGF = insulin-like growth factor; BMP = bone morphogenetic proteins; PGE2 = prostaglandin E2; PIIANP = type IIA collagen N-propeptide; CII = type II procollagen carboxy-propeptide.

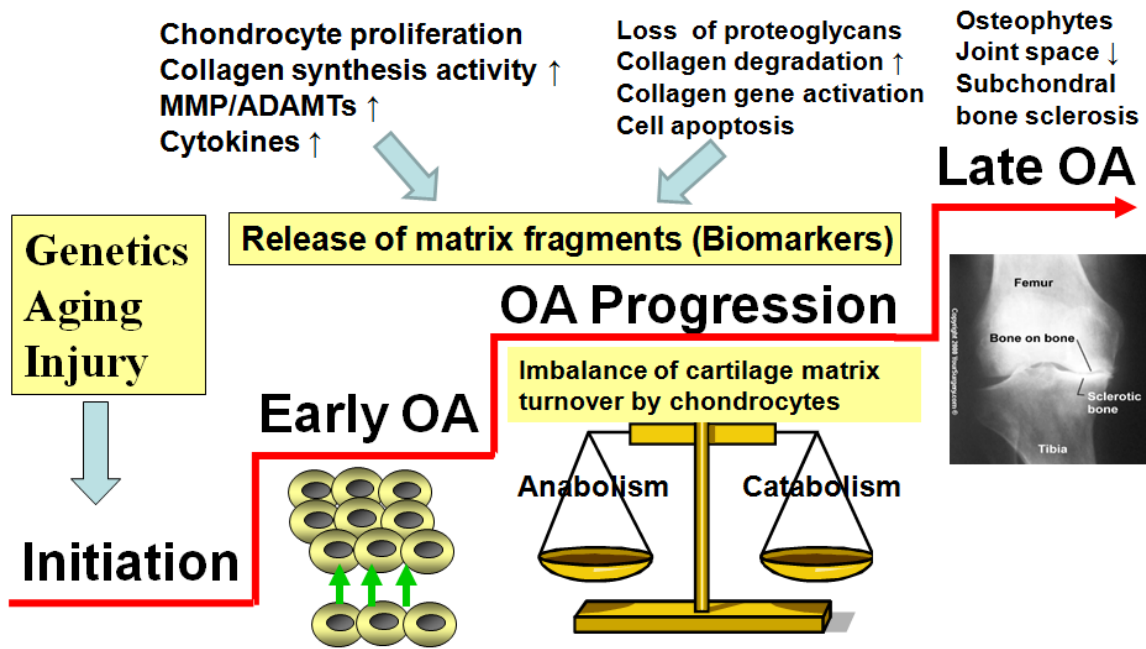


Figure 1.5: Initiation and progression of OA. Cellular and morphological changes are listed above. Initiating factors are listed in the Lt box. Biomarkers are released during the OA process. MMP = matrix metalloproteinase; ADAMTS = ADAM metalloproteinase with thrombospondin type 1

1.1.1.5 Treatment of OA

Although extensive research and drug discovery programs would like to identify the initiating events to halt cartilage destruction in OA, current effective treatment strategies for OA have not improved much over decades. A major challenge is to identify biochemistry markers or genetic initiating factors to recognize individuals with high OA risk, which can be used for future targeted interventions rather than symptom-modifying drugs.

1.2 OA Biomarkers

Progressive loss of articular cartilage associated with subchondral bone remodeling, and synovial changes major features of OA. Changes in joint space width (JSW) estimated by radiography remains the “gold” standard; however, radiography to visualize the degenerative changes in the joint can be observed only at an advanced stage of OA, in which joint tissue damage is irreversible. Therefore, these late phenotypes are relatively insensitive at an early detection stage of the OA. Furthermore, radiography only provides an anatomical view of structure that has already occurred, rather than monitoring the dynamic changes of joint tissues.

Biomarkers which reflect dynamic and quantitative changes in joint tissue remodeling have been proposed as an alternative method to detect OA development and progression (Garnero 2006). A biochemical marker is usually

regarded as a distinct molecule or fragment of cartilage matrix which is released into the blood or body fluids during the process of tissue turnover. Because type II collagen is the most abundant protein in the cartilage, recent biomarkers are mainly based on detecting balance between its synthesis and degradation (such as CII, PIIANP, C₂C) (Garnero, Rousseau et al. 2000). Other biomarkers for OA focus on non-collagenous proteins (such as HA, COMP) and inflammation markers (such as Hs-CRP) related to the OA pathological process.

1.2.1 BIPED classification

To describe the potential uses of biomarkers, the BIPED scheme (**b**urden of disease, **i**nvestigative, **p**rognostic, **e**fficacy of intervention and **d**iagnostic) was proposed in 2006 to classify OA markers (Bauer, Hunter et al. 2006). This classification sub-classifies current potential OA biomarkers into five categories according to whether these markers can provide information on the burden of disease, being investigative, prognosis prediction, reflection of effective treatment, and disease diagnosis (Bauer, Hunter et al. 2006). Summaries of hypothesis and classification of BIPED are shown in Table 1.2 and Figure 1.6

Table 1.2 Selected osteoarthritis biomarkers according to the BIPED classification and assumed pathological process

Tissue	Molecule	Markers of synthesis	Markers of degradation	BIPED classification
Cartilage	Type II collagen	PIIANP CPII		B,P,D P,E,D P,E,D B,P,D
	Non-collagenous proteins		C₂C COMP	
Synovium	Non-collagenous proteins	HA		B,P
Systemic inflammation		Hs-CRP		

PIIANP = type IIA collagen N-propeptide; CPII = type II procollagen carboxy-propeptide; HA = Hyaluronan; HS-CRP = High-sensitivity C - reactive protein; C2C = neoepitope from cleavage of CII; COMP = Cartilage Oligomeric Matrix Protein; B = Burden of disease markers; D = Diagnostic markers; E = Efficacy of intervention markers; P = Prognostic markers.

BIPED

- **B**urden of Disease
- **I**nvestigative
- **P**rognostic
- **E**fficacy of Therapeutic Intervention
- **D**iagnostic

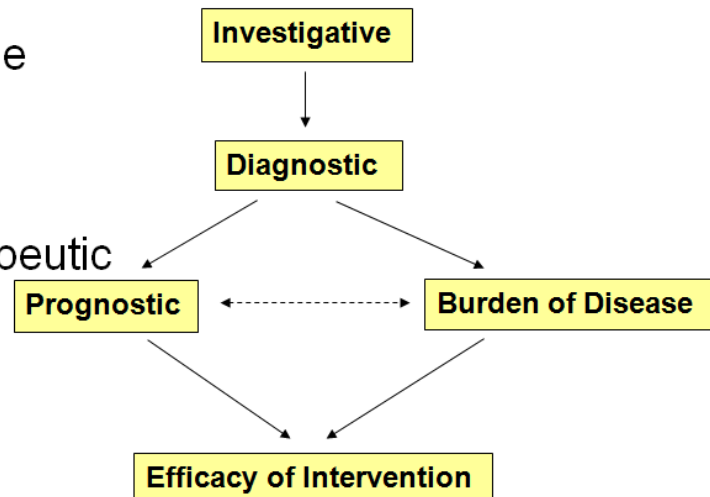


Figure 1.6: Hypothetical development of OA biomarkers. Adapted from Bauer, D. C., D. J. Hunter, et al. (2006). "Classification of osteoarthritis biomarkers: a proposed approach." Osteoarthritis Cartilage 14(8): 723-7.

1.2.1.1 Burden of disease markers

“Burden of disease” markers evaluate the severity or extent of OA, typically at a single point in time, among affected individuals. The compared phenotypes can be severity within a particular joint, and/or severity of a number of joints involved. To establish such marker classification, cross-sectional study design was performed based on OA individuals and normal controls in clinical trials.

1.2.1.2 Prognostic markers

As indicated by Bauer et al., the major feature of a Prognostic marker is able to predict the future onset of OA among normal people at a baseline or the progression of OA among affected individuals. The evaluation of Prognostic markers requires longitudinal studies representing associations between markers and radiographic phenotypes.

1.2.1.3 Efficacy of intervention markers

“Efficacy of intervention” markers mostly provide information about the treatment effect of the given markers for those with OA or at a high risk of developing OA. Efficacy of intervention markers should be measured prior to therapy and evaluated with appropriated OA phenotypes.

1.2.1.4 Diagnostic markers

“Diagnostic” markers are defined as having an ability to differentiate individuals as either diseased or non-diseased. Usually, the markers should be evaluated by comparison with the radiographic OA gold standard (Kellgren-Lawrence KL grade ≥ 2). Most studies use case-control designs in a cross-sectional population.

1.2.1.5 Investigative markers

“An Investigative” marker is one for which there is scanty evidence to allow its inclusion into one of the existing categories (diagnostic, prognostic, burden of disease, treatment effect). This potential OA biomarker therefore should be further investigated and validated for its role in OA.

1.2.2 Reasons for Using these Seven Biomarkers to Detect OA

Type II collagen related epitopes: PIIANP (type IIA collagen N-propeptide), CPII (type II procollagen carboxy-propeptide), and C₂C (neoepitope from cleavage of CII).

Type II collagen (CII) provides the major part of the organic components (15%-22%) (Heinegard and Saxne 1991). It is a triple helical protein that shapes the fibrillar structure of the extracellular matrix of cartilage and has been presumed to mainly reflect cartilage processes. The triple helical protein consists of three $\alpha 1$ chains, and the linear N- and C-telopeptides (Fig 1.7). In humans, there are two splicing alternative forms of type II (type IIA and type IIB

procollagen) depending on whether it is with or without a 69 amino acid sequence coded by exon 2 in the N-propeptide (Rousseau, Sandell et al. 2004).

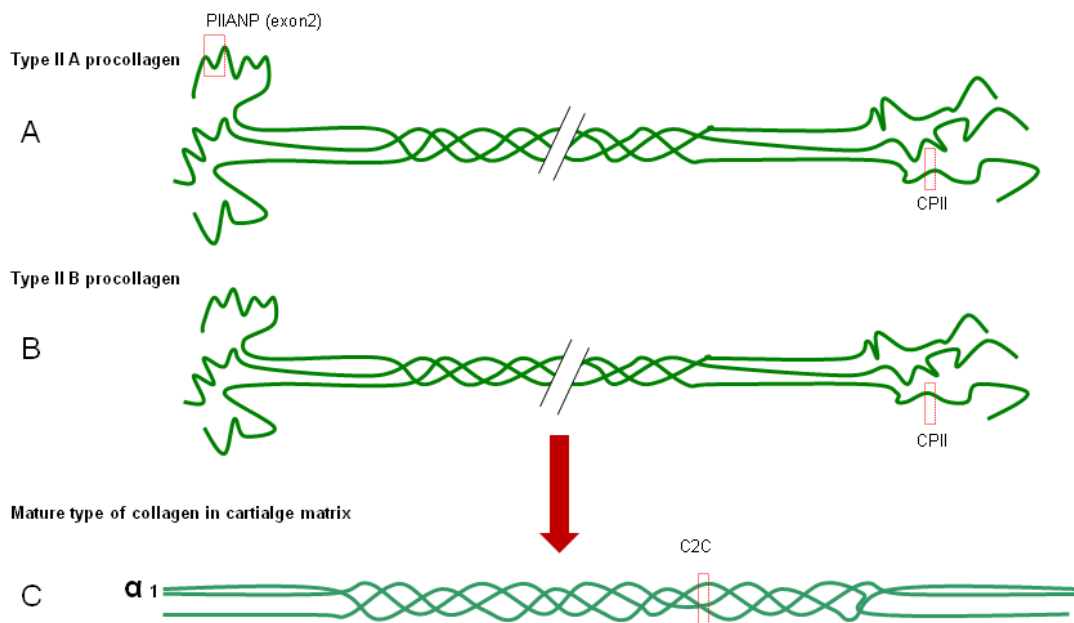


Figure 1.7: Diagram of the type II collagen molecule. Two forms of type II procollagen exist. A, procollagen IIA, which includes a peptide of 69 amino acids coded by exon 2 in the N-propeptide. B, procollagen IIB, in which this peptide is absent. C. the type II collagen neopeptides C2C. CPII exists in both forms of type II procollagen. Modified from Charni-Ben Tabassi, N. and P. Garnero (2007). "Monitoring cartilage turnover." Curr Rheumatol Rep 9(1): 16-24.

1.2.2.1 Markers of Cartilage Synthesis

1.2.2.1.1 PIIANP (type IIA collagen N-propeptide)

Procollagen IIA is primarily expressed during development and can be re-expressed in OA cartilage (Sandell, Morris et al. 1991; Aigner, Zhu et al. 1999). N-terminal propeptide is removed by proteases during the secretion to extracellular cartilage matrix space from chondrocyte (Ryan and Sandell 1990). By detecting N-propeptide from blood or synovial fluid, the index of type II collagen synthesis can be measured. Most of the previous studies have demonstrated that serum PIIANP was decreased in patients with knee OA compared to healthy sex and age-matched controls (Garnero, Ayrat et al. 2002; Rousseau, Sandell et al. 2004; Rousseau, Zhu et al. 2004). Another study also showed a good prediction of radiologic progression by combining PIIANP (cartilage synthesis) and a marker of cartilage degradation CTX-II (C-telopeptide of type II collagen) (Garnero, Ayrat et al. 2002).

1.2.2.1.2 CPII (type II procollagen carboxy-propeptide)

CPII is also subjected to specific enzymatic cleavage before integrating type II collagen into mature fibrils (Peltonen, Halila et al. 1985). The released CPII is composed of three 35 kDa proteins interconnected by inter-chain disulfide bond (Niyibizi, Wu et al. 1987). CPII levels were lower in the serum of OA individuals than the healthy control participants (Nelson, Dahlberg et al. 1998; Otterness,

Swindell et al. 2000). Synovial fluid CPII also was found to be positively correlated with early OA disease stages (Kobayashi, Yoshihara et al. 1997). In brief, research demonstrated that type II collagen synthesis was elevated in the early stages of OA, but the levels declined in the advanced stages (Lohmander, Yoshihara et al. 1996; Nelson, Dahlberg et al. 1998).

1.2.2.2 Markers of Cartilage Degradation

1.2.2.2.1 C₂C (neoepitope from cleavage of CII)

C₂C is a neoepitope identified at the C-terminus of the $\frac{3}{4}$ length fragment of type II collagen. The cleavage of type II collagen molecules is caused by the collagenase subfamily known as metalloproteinases (MMPs), collagenases 1, 2, 3 and membrane type 1-MMP (such as MMP-1, MMP-8, MMP-13, and MMP-14) (Billinghurst, Dahlberg et al. 1997). These collagenases mediated cleavage between Gly794 and Leu795, which results in a $\frac{3}{4}$ length fragment and a $\frac{1}{4}$ length fragment (Mitchell, Magna et al. 1996). After the cleavage by MMPs, the triple helix of type II collagen fragments uncoils and enables denatured fragments to further degradation by a diverse of proteolytic enzymes. Recently, antibodies recognizing different type II collagen fragments have been identified (Billinghurst, Dahlberg et al. 1997; Poole, Ionescu et al. 2004; Charni, Juillet et al. 2005). C₂C monoclonal antibody was developed by a commercially available competitive ELISA assay using the synthetic peptide CGGE787GPOGPQG794 (O denotes

hydroxyproline) (Poole, Ionescu et al. 2004). Clinically, C₂C has revealed a significant correlation with cartilage volume by magnetic resonance imaging with knee OA (King, Lindsey et al. 2004). C₂C has also been shown to be decreasing in patients with rheumatoid arthritis after treatment of infliximab (Visvanathan, Marini et al. 2007).

1.2.2.3 Non-collagenous Proteins

1.2.2.3.1 COMP (Cartilage Oligomeric Matrix Protein)

COMP is a 524 kDa homopentameric non-collagenous glycoprotein derived from cartilage and also found in ligaments and tendons (Muller, Michel et al. 1998). Recent *in vitro* studies have shown that COMP can interact with collagens I, II, IX, fibronectin, and all matrilins (Rosenberg, Olsson et al. 1998; Holden, Meadows et al. 2001; Di Cesare, Chen et al. 2002; Mann, Ozbek et al. 2004), and that COMP can bind to collagens I, II, and IX with high affinity (Thur, Rosenberg et al. 2001). In humans, studies reported elevations in serum COMP with the presence and severity of radiographic knee OA, bilateral radiographic knee OA, the presence of concomitant radiographic hip and knee OA, and the number of knees and hips with radiographic evidence of OA (Clark, Jordan et al. 1999; Vilim, Olejarova et al. 2002). Another study also found an association of elevated serum COMP levels with clinical signs and symptoms of arthritis in the

absence of radiographic hip and knee OA suggesting that serum COMP may be useful as a biomarker of pre-radiographic joint pathology (Dragomir, Kraus et al. 2002). In animals, murine OA results in upregulation of COMP mRNA production, implying that synovial fluid COMP levels may represent tissue degradation (Salminen, Perala et al. 2000).

1.2.2.3.2 HA (Hyaluronan)

HA is a glycosaminoglycan of connective tissue found as a major constituent in cartilage, synovium and synovial fluid. Although HA is not joint specific, studies have shown a strong association exists between serum HA levels and presence and severity of radiographic OA (Pavelka, Forejtova et al. 2004; Elliott, Kraus et al. 2005). Also, HA levels are predictive of progressive joint pathology (Sharif, George et al. 1995). These associations with OA in varying stages of disease suggest it may be useful as a biomarker in OA studies.

1.2.2.4 Inflammatory marker

1.2.2.4.1 Hs-CRP (High-sensitivity C-Reactive Protein)

CRP is an acute-phase protein produced by hepatocytes in response to infectious and non-infectious inflammation. It consists of five single subunits, which are noncovalently linked and assembled as a cyclic pentamer with a molecular weight of 110-140 kDa. Hs-CRP has been regarded as a trustworthy

inflammation marker. Recently, low-grade inflammation has been increasingly recognized as a component of osteoarthritis(Sharif, Shepstone et al. 2000). Many reports on a possible relationship between serum Hs-CRP and OA have been identified (Spector, Hart et al. 1997; Jordan, Luta et al. 2002; Sowers, Jannausch et al. 2002). However, several studies also suggested serum Hs-CRP was influenced by obesity, body mass index, and cardiovascular comorbidities, which may confound the interpretation of results.

1.2.2.5 Risk Factor of Aging

1.2.2.5.1 GSP (Glycated serum protein)

GSP is an intermediate to advanced glycation end product (AGE) formation. AGEs are formed by non-enzymatic reactions in the process of post-translational modification, and have been involved in the aging process and the pathogenesis of several diseases, including rheumatoid arthritis (RA) and diabetes. A recent study has shown that accumulation of AGEs is a potential risk factor for OA (DeGroot, Verzijl et al. 2004). Verzijl N et al also showed that AGE crosslinking may result in pathologic stiffness of cartilage *in vitro* (Verzijl, DeGroot et al. 2002). Senolt et al reported an increased serum concentration of pentosidine, a form of AGE, in patients with knee OA (Senolt, Braun et al. 2005). Therefore, GSP may be a candidate molecular marker to detect OA.

1.3 OA Genetics

Heberden's OA (a form of OA of distal interphalangeal joints) was noted in 1940s. This nodal type of OA has been found predominates in women and inherited as a dominant trait (Stecher 1950). In the past ten years, twin-pair and segregation studies have been conducted and revealed that OA had a major genetic component (Spector, Cicuttini et al. 1996; Felson, Couropmitree et al. 1998; Hirsch, Lethbridge-Cejku et al. 1998). Nevertheless, it is in recent years that more in-depth genetic studies were designed and have identified several candidate regions and candidate genes. A number of strategies can be used to identify OA susceptibility genes. These include family genome-wide linkage scans, candidate gene studies based on biological clues, whole-genome association scans and gene expression studies.

1.3.1 Genome-wide Linkage Studies of OA

Linkage analysis is a traditional approach for mapping genes. The definition of linkage is that two linked loci are physically connected on the same chromosome at a distance that is measured at less than 50% genetic recombination. Genetic recombination allows genetic diversity in a population by the exchange of DNA between chromatids via the stage of chromosomes crossover during meiosis. These exchange genetic regions of recombination can

be transmitted or segregated to offspring by parents. Familial linkage takes place when Mendel's third law is violated, indicating that two or more genetic loci co-segregate in a family. Genetic linkage is measured in centimorgans (cM) – a unit of percentage of recombination - with an additive characteristic. In general, 1cM is roughly equal to 1MB (megabase) because recombination does not usually occur randomly. For linkage analysis, recombination can be scored from informative meioses in families. The likelihood-based method for scoring recombinants in families was described by Morton in 1955 (Morton 1955). The sequential linkage test is used to calculate a LOD score which stands for the \log_{10} of the odds for linkage. As suggested by Morton, a LOD score greater than 3.0 can be considered as the threshold in which linkage is detected, indicating odds of 1000 to 1 in favor of linkage. LOD scores less than -2 could be regarded as no evidence of linkage. However, for complex diseases, many genetic studies also yield susceptibility genes from a LOD score between 1.5 and 3.0, which was also defined as suggestive significance.

Through investigating the co-segregation of DNA markers with inheritance of a given trait in families, genome-wide linkage scan allows examination of the entire genome in order to have a context in which to place candidate gene association studies. Microsatellites and single-nucleotide polymorphisms (SNPs) are the genetic markers most used for linkage studies. To date, five genome-wide linkage scans have been published, relying on small families of affected

relatives or twins collected in the United States (Demissie, Cupples et al. 2002; Hunter, Demissie et al. 2004), United Kingdom (Chapman, Mustafa et al. 1999; Loughlin, Mustafa et al. 1999; Livshits, Kato et al. 2007), Finland (Leppavuori, Kujala et al. 1999), and Iceland (Ingvarsson, Stefansson et al. 2001; Stefansson, Jonsson et al. 2003). In general, the U.K. scan was used to identify OA candidate loci by using phenotypes of hip or knee OA with joint replacement. Other genome-wide scans were performed on patients with hand OA. In brief, if the proportion of alleles which is identical by descent share at a given marker is higher in affected family members than expected, we can conclude that this marker is close to an OA-related candidate gene.

In the previous five linkage studies, OA phenotypes were defined by X-ray evidence, physical example, or joint replacement, which detect late stages of OA. To date, no study using OA-related biomarkers identifies early stage of OA candidate genes or loci. A summary of the chromosomal regions identified by previous linkage analyses is listed in Table 1.3.

Table 1.3 Chromosomal regions identified from previous genome-wide linkage scans

Chromosome	Cytogenetic location	Country	Phenotypes
1	1p32-p22	USA	Hand OA
2	2q12-2q21	Finland	Hand/knee/hip OA
	2q31.1-2q34	UK	Hip OA
	2p23.2-2p16.2	USA, Iceland	Hand OA
3	3p22.2-3p14.1	Iceland	Hand OA
4	4q26-4q32.1	Finland, Iceland	Hand OA
6	6p21.1-6q15	UK	Hip OA
7	7q34-7q36.3	USA	Hand OA
	7p15-7p21	Finland	Hand OA
11	11p12-11q13.4	UK	Hip OA
13	13q33.1-13q34	USA	Hand OA
15	15q21.3-15q26.1	USA	Hand OA
16	16p13.1-16q12.1	UK, Iceland	Hip OA
	16q22.1-q23.1	UK	Knee/hip OA
19	19q13	USA, UK	Hand OA

Modified from Valdes, A. M. and T. D. Spector (2008). "The contribution of genes to osteoarthritis." Rheum Dis Clin North Am 34(3): 581-603.

1.3.2 Genetic Association Studies and Candidate Genes Approach of OA

Candidate gene studies are initiated by choosing one or more genes based on the previous knowledge involving the pathogenesis of OA. In general, these studies compare genotype and allele frequencies between OA cases and controls. The most selective genes are from genes encoding extracellular matrix components (Kizawa, Kou et al. 2005; Mototani, Mabuchi et al. 2005; Kamarainen, Solovieva et al. 2006), genes encoding for proteins affecting bone density (Valdes, Van Oene et al. 2006), and genes influencing catabolic and anabolic cytokines (Stern, de Carvalho et al. 2003; Pola, Papaleo et al. 2005). However, just like other complex diseases, OA association studies often face many shortcomings: small sample sizes and limited investigation of genes (Peach, Carr et al. 2005). Therefore, further replication of these candidate genes from different populations and further validation of functional studies for these candidate genes are crucial (Ikegawa 2007). Although a total number of over 60 candidate genes were investigated since the late 1990s, only limited OA candidate genes have been replicated from different populations or validated from functional studies such as FRZB, ASPN, and BMP2 etc. (Loughlin, Dowling et al. 2004; Kizawa, Kou et al. 2005; Valdes, Van Oene et al. 2006).

1.3.3 Genome-wide Association Study (GWAS) of OA

The GWAS study compares gene-variant prevalence between cases and controls using a genome-wide comparison, so that the previous assumptions of OA-candidate genes based on the biological relevance can be avoided. Rather than having a genetic association study using a candidate approach, more genetic markers are typed through coverage of the whole genome. Therefore, if a genetic marker (or SNP) is identified to be significantly associated with OA, this genetic marker (or SNP) is actually closer to, and even involved with OA. Till now, four large GWAS have been reported and four candidate genes were found (CALM1, LRCH1, PTGS2 and DVWA) (Mototani, Mabuchi et al. 2005; Spector, Reneland et al. 2006; Miyamoto, Shi et al. 2008; Valdes, Loughlin et al. 2008). A variant in the CALM1 gene was associated with OA in the Japanese population (Mototani, Mabuchi et al. 2005), but this variant cannot be further replicated in the UK population (Loughlin, Sinsheimer et al. 2006). Likewise, a variant in the LRCH1 gene was significantly associated with radiographic knee OA in Caucasians (Spector, Reneland et al. 2006), but this SNP failed to be replicated in Asians (Jiang, Shi et al. 2008). PTGS2 and DVWA may need to be further validated among different races in the near future.

1.3.4 Quantitative Trait Linkage Analysis

The purpose of many gene discovery studies is to find genes that represent an increased susceptibility to a particular disease. The clinical endpoint of the

trait can be the presence or absence of disease. However, the clinical endpoints also can be a wide range of values, which can be quantitative traits actually reflecting the disease status (Ott 1995). Therefore, an alternative strategy to facilitate identification of susceptibility genes for the disease is to use subclinical phenotypes (or endophenotypes) as the clinical endpoints. These endophenotypes may bring one much closer to the underlying pathophysiological mechanism of disease in order to identify the underlying loci or genes (Mackay 2001) (Figure 1.8).

In brief, linkage analysis uses the parametric approach based on knowing genetic models (such as autosomal dominance, autosomal recessive and X-linked traits). As to complex diseases with multi-factorial factors, more robust nonparametric methods have been developed. Variance component linkage analysis is one of the nonparametric approaches created by Amos (Amos 1994) and it can be implemented in the package of SOLAR[®] (Sequential Oligogenic Linkage Analysis Routines) (Almasy and Blangero 1998). The trait value for each individual can be modeled as $y = \mu + \sum \beta_j v_{ij} + g_i + e_i$, where y is the phenotypic value for the individual, β_j is the regression coefficient for the j th covariate, v_{ij} represents the value of covariate j for the i th individual and g and e represent the additive genetic effects and environmental effects, respectively.

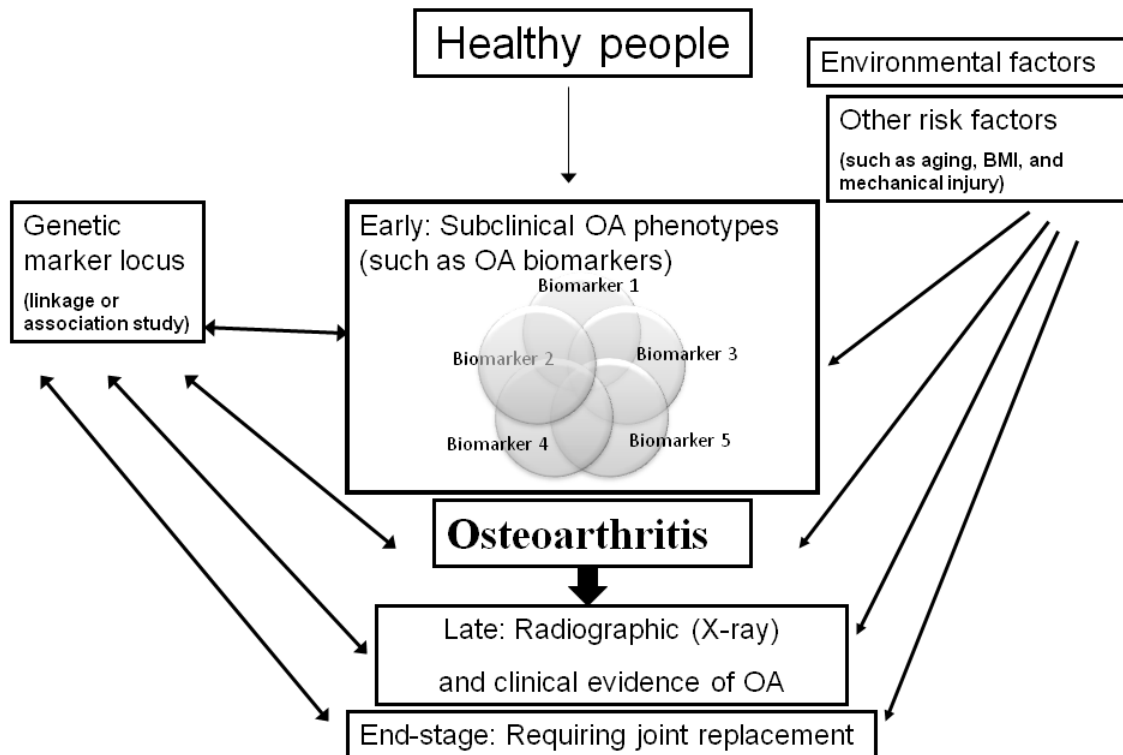


Figure 1.8 Pathways to Osteoarthritis. Subclinical OA phenotypes using OA biomarkers may detect early OA candidate genes. Adapted from Chen HC, Shah SH, Li YJ, Nelson S, Haynes C, Johnson J, Stabler T, Hauser ER, Gregory SG, Kraus WE, Kraus VB. American Society of Human Genetics 2008: C2051 (poster).

1.4 Hypermobility and OA

Joint hypermobility is widely prevalent in all communities, but its clinical effects remain poorly understood. Joint hypermobility differs noticeably between sexes and is two to three times more prevalent among females than males (Larsson, Baum et al. 1987). Also, joint hypermobility is more prevalent among Asian and African races than among Caucasians (Grahame 1999). A female twin study also suggested that joint hypermobility has a strong genetic component (Hakim, Cherkas et al. 2004). Joint hypermobility may come from a series of well-defined monogenic disorders such as Marfan's syndrome, Ehlers-Danlos syndrome (EDS), osteogenesis imperfect, benign joint hypermobility syndrome (BJHS), pseudoachondroplasia (PSACH), and multiple epiphyseal dysplasia (MED) (Zweers, Hakim et al. 2004; Bravo and Wolff 2006). It may, however, occur among normal individuals who do not have the above clinical symptoms (Birrell, Adebajo et al. 1994). Indeed, joint hypermobility is an asset to musicians, dancers, and athletes (Larsson, Baum et al. 1993). OA has been reported to occur more often in patients with EDS/BJS and Marfan's syndrome (Zweers, Hakim et al. 2004). Pseudoachondroplasia and multiple epiphyseal dysplasia are also characterized with early-onset OA (Briggs and Chapman 2002). However, some studies have also shown that a joint hypermobility trait may decrease OA risk (Dolan, Hart et al. 2003; Kraus, Li et al. 2004). Examples of mutations in extracellular matrices (ECMs) with hypermobility phenotypes are listed in Table 1.4.

Table 1.4 Examples of mutations in ECM proteins with joint hypermobility phenotypes

ECM component	Gene(s)	Inheritance	Major tissue(s) affected	Disease
COMP	<i>COMP</i>	AD	Cartilage, ligaments	MED, PSACH
Collagen 1	<i>COL1A1, COL1A2</i>	AD	Skin, joints	EDS, type VII
	<i>COL1A1, COL1A2</i>	AD	Bone	OI
Collagen V	<i>COL5A1, COL5A2</i>	AD	Skin, joints	EDS, type I,II
Collagen IX	<i>COL9A1, COL9A2,</i>	AD	Cartilage	MED
	<i>COL9A3</i>			
Fibrillin 1	<i>FBN1</i>	AD	Skeleton, eyes, cardiovascular	Marfan syndrome
Matrilin 3	<i>MTN3</i>	AD	Cartilage	MED
Tenascin XB	<i>TNXB</i>	AR	Skin	EDS-like syndrome
		AD	Skin	EDS, type III

AD = autosomal dominant; AR = autosomal recessive; COMP = cartilage oligomeric matrix protein; ECM = extracellular matrix; MED = multiple epiphyseal dysplasia; PSACH = pseudoachondroplasia; EDS = Ehlers-Danlos syndrome; OI = osteogenesis imperfecta

1.5 Background of CARRIAGE Family

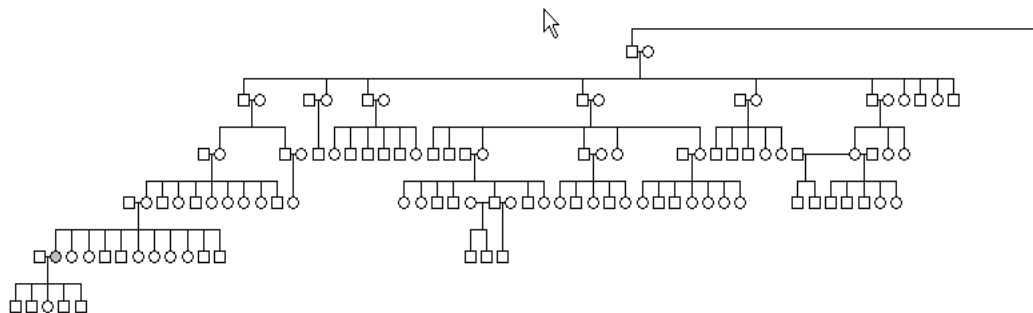
The CARRIAGE (CARolinas Region Interaction of Aging, Genes and Environment) family described here is of mainly mixed African American and Native American ancestry. It is one of the most extensively pedigreed existing families in the United States comprising nine generations with 3357 pedigreed members, and originating from one founder born in the 1700s. This original forefather lived from 1773-1862 and had 10 children (9 males and 1 female) (Figure 1.9). Caucasian lines later intermarried into this family, causing a highly multi-ethnic pedigree. Ascertainment of 350 family members was conducted during three family reunions from 2002-2006. The characteristics of the CARRIAGE family pedigree is summarized in Table 1.5.

Figure 1.9 Main family pedigree: Decendents of the original Family forefather who lived 1773-1862.

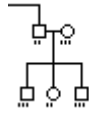
The forefather of the Family had 10 children (9 sons and 1 daughter).

1. Son #1 had 6 children (3 males, 3 females)

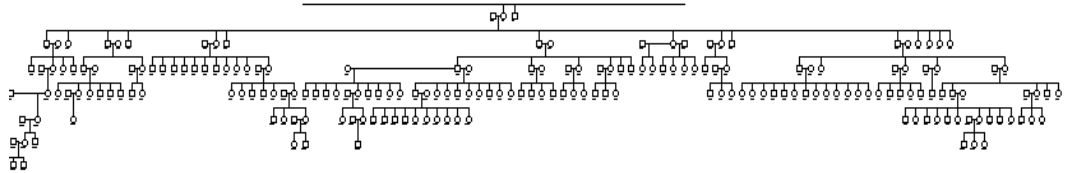
A. Grandson #1:



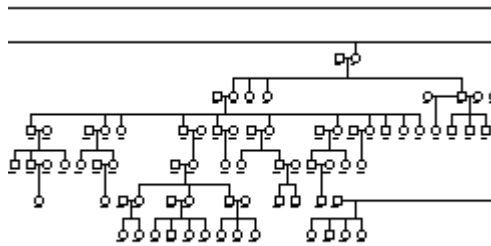
- B. Grandson #2: no documented offspring
- C. Grandson #3 had 3 children (2 sons, 1 daughter):



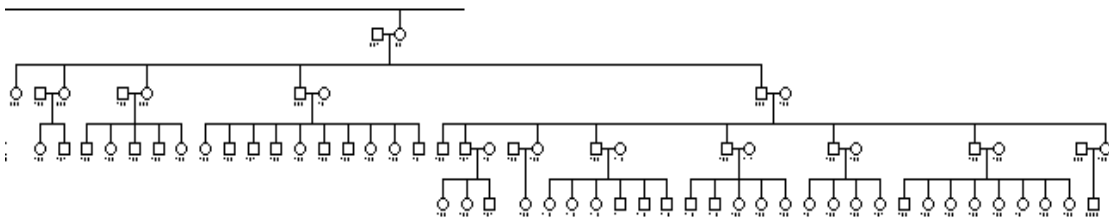
- D. Granddaughter #1 had 15 children (9 sons, 6 daughters):



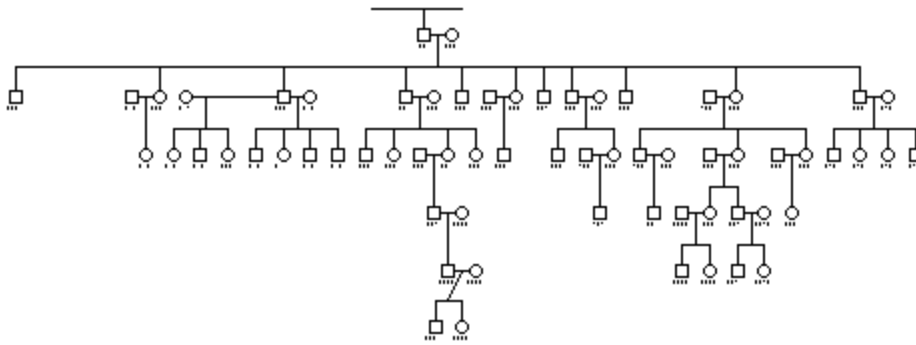
- E. Granddaughter #2 had 4 children (1 son, 3 daughters):



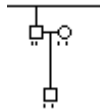
- F. Granddaughter #3 had 5 children (2 sons, 3 daughters):



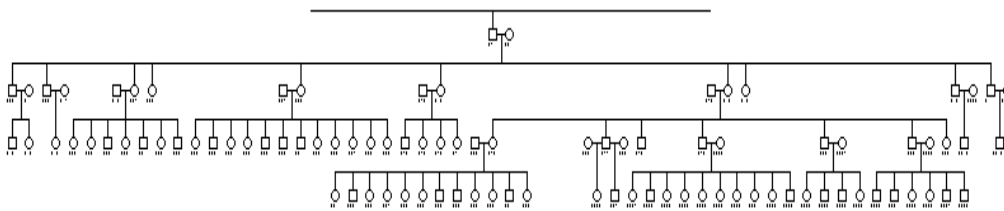
- 2. Son #2 had 8 children (4 sons, 4 daughters)
 - A. Grandson #1 had 11 children (8 sons, 3 daughters)



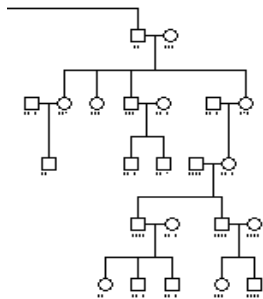
B. Grandson #2 had 1 child:



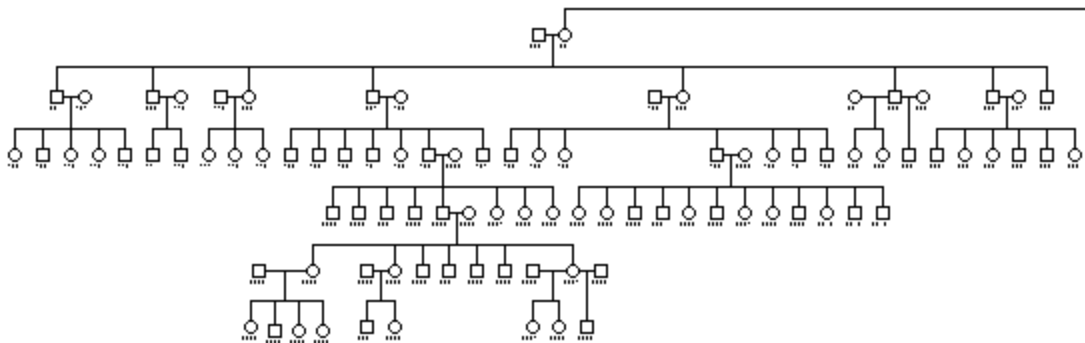
C. Grandson #3 had 10 children (4 sons, 6 daughters):



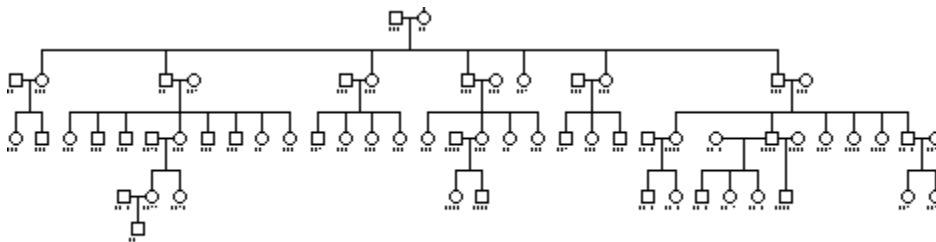
D. Grandson #4 had 4 children (1 son, 3 daughters):



E. Granddaughter #1 had 8 children (6 sons, 2 daughters):

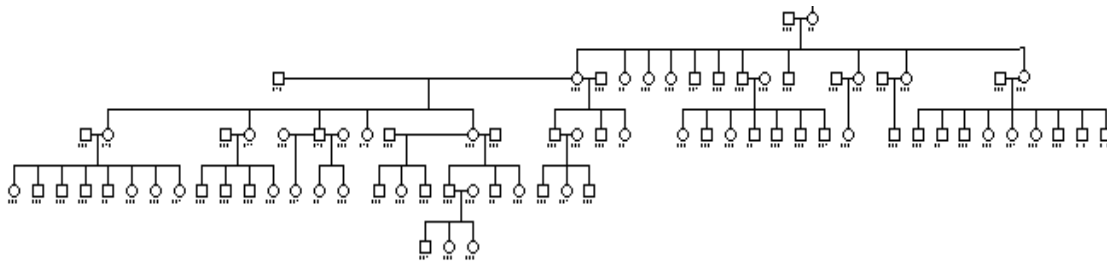


F. Granddaughter #2 had 7 children (3 sons, 4 daughters):

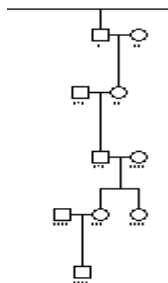


G. Granddaughter #3 had no documented offspring.

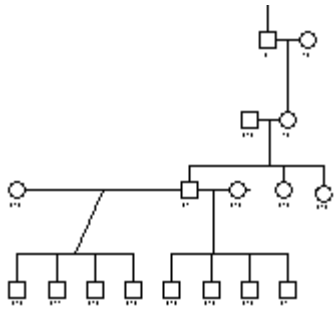
H. Granddaughter #4 had 11 children (4 sons, 7 daughters):



3. Son #3 had 1 child (daughter):



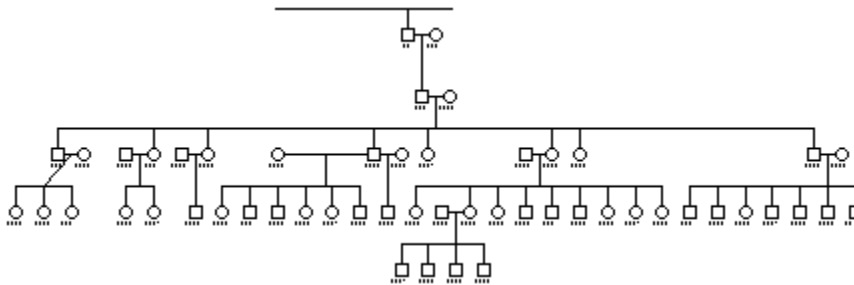
4. Son #4 had 1 child (daughter):



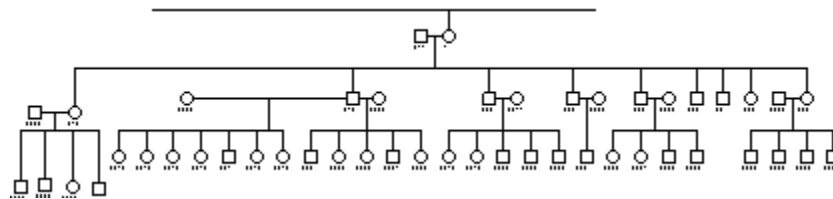
5. Son #5 had 9 children (2 sons, 7 daughters):

A. Grandson #1 had no documented offspring;

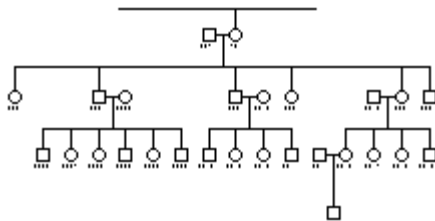
B. Grandson #2 had 1 son:



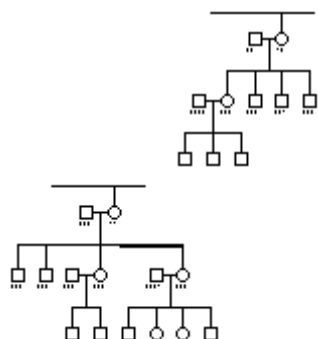
C. Granddaughter #1 had 9 children (6 sons, 3 daughters):



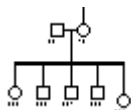
D. Granddaughter #2 had 6 children (3 sons, 3 daughters):



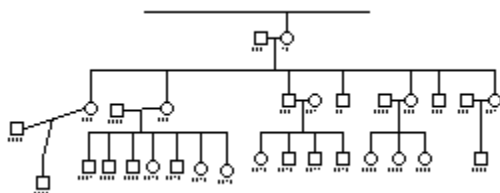
- E. Granddaughter #3 had 4 children (3 sons, 1 daughter):
- F. Granddaughter #4 had 4 children (2 sons, 2 daughters):



- F. Granddaughter #5 had 5 children (3 sons, 2 daughters):



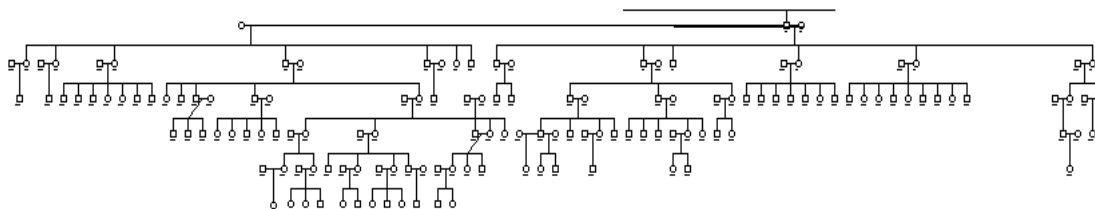
- G. Granddaughter #6 had 7 children (3 sons, 4 daughters):



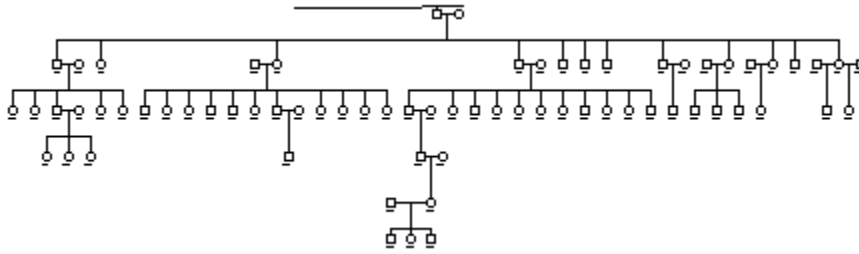
- H. Granddaughter #7 had no documented offspring.

6. Son #6 had 20 children (12 sons, 8 daughters):

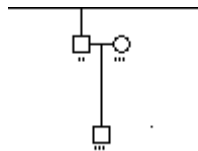
- A. Grandson #1 had 13 children by 2 wives (7 daughters, 6 sons):



- B. Grandson #2 had 12 children (7 sons, 5 daughters):

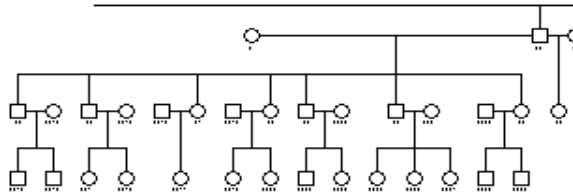


C. Grandson #3 had 1 son:



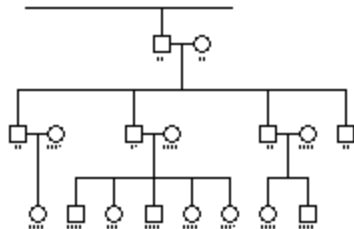
D. Grandson #4 had no documented offspring.

E. Grandson #5 had 8 children by two wives (4 sons, 4 daughters):



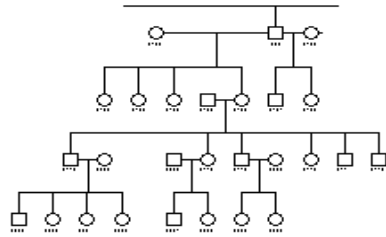
F. Grandsons #6-9 had no documented offspring.

G. Grandson #10 had 4 sons:

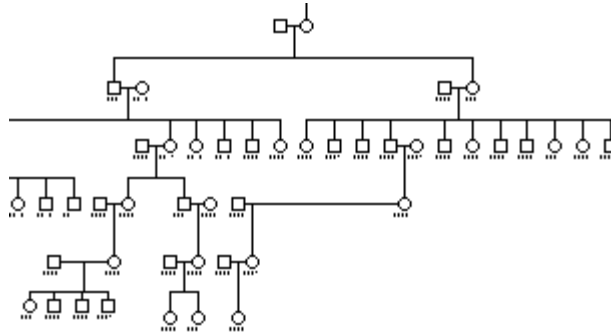


H. Grandson #11 had no documented offspring.

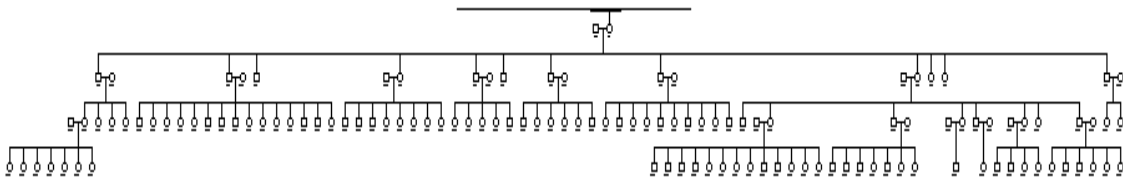
I. Grandson #12 had 15 children by 3 wives (5 sons, 10 daughters, third wife not shown):



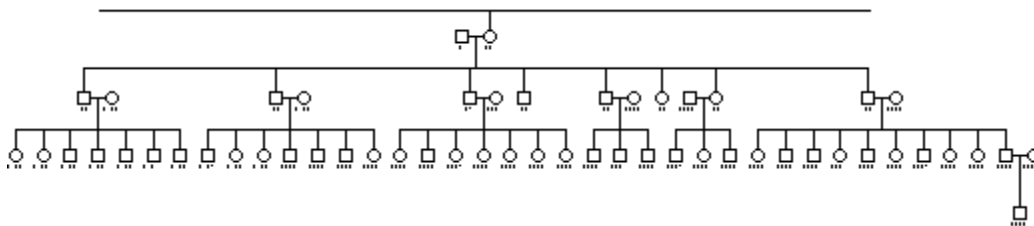
J. Granddaughter #1 had 2 children (1 son, 1 daughter):



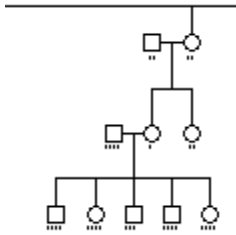
K. Granddaughter #2 had 12 children (9 sons, 3 daughters):



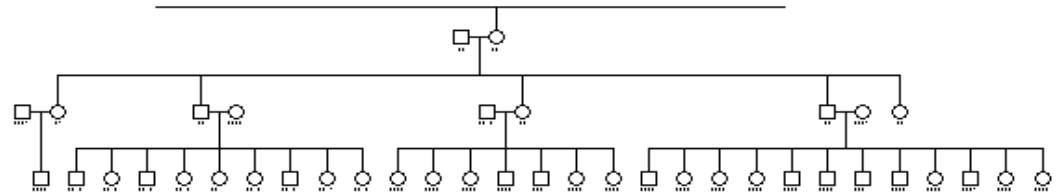
L. Granddaughter #3 had 8 children (6 sons, 2 daughters):



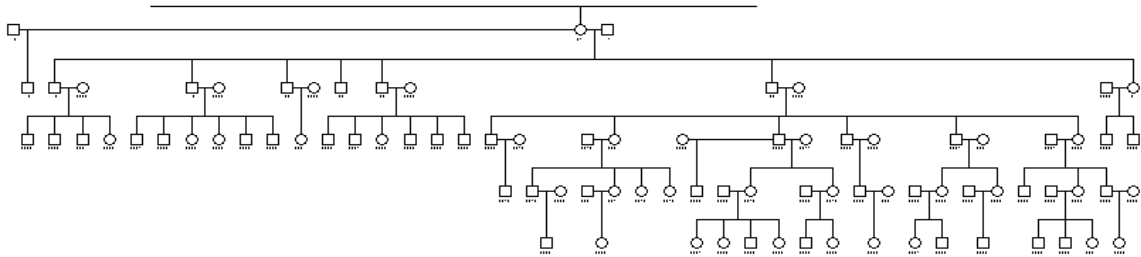
M. Granddaughter #4 had 2 daughters:



N. Granddaughter #5 had 5 children (2 sons, 3 daughters):



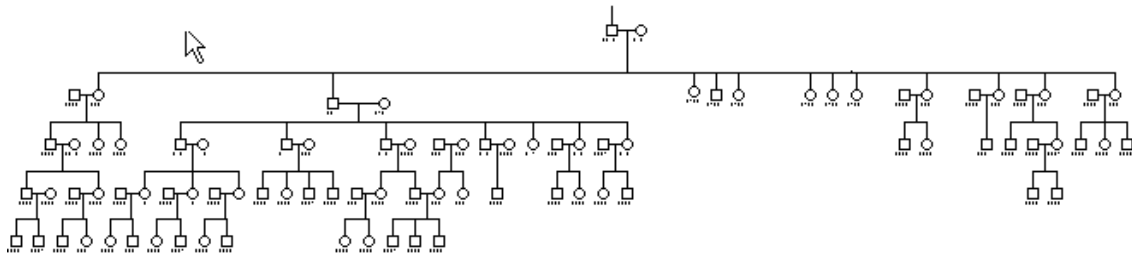
O. Granddaughter #6 had 8 children by two husbands (8 sons):



P. Granddaughter #7 and #8 had no documented offspring.

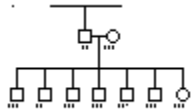
7. Daughter #1 had 11 children (6 sons, 5 daughters):

A. Grandson #1 had 12 children (2 sons, 10 granddaughters):



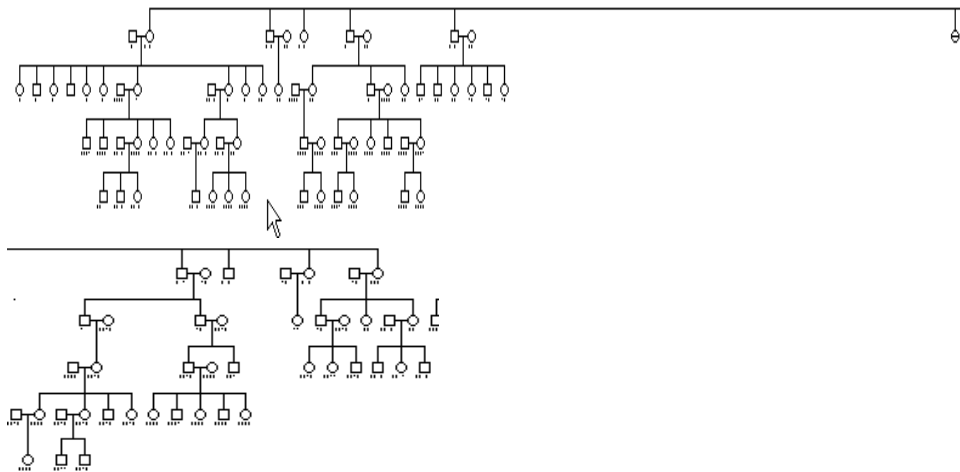
B. Grandson #2 had no documented offspring.

C. Grandson #3 had 7 children.



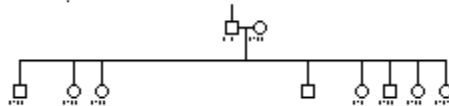
D. Grandsons #4-#6 had no documented offspring.

E. Granddaughter #1 had 10 children (6 sons, 4 daughters):



D. Granddaughters #2-#5 had no documented offspring.

8. Son #7 had 8 children (3 sons, 5 daughters).



9. Son #8 had 9 children (5 sons, 4 daughters).

10. Son #9 had 8 children (4 daughters, 4 sons).

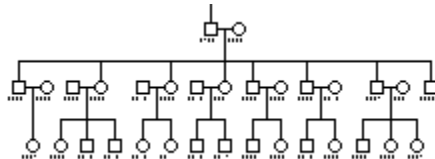


Table 1.5 Characteristics of the CARRIAGE Family Pedigree

Relationship	No. of Pairs
Parent-offspring	127
Full-Siblings	78
Half-Siblings	8
Cousin	258
Avuncular	189
Grandparent-grandchild	19
3rd degree	79
4th degree	648
\geq 5th degree	1,470

1.6 Hypotheses and Aims

Specific Aim 1. Complete this multigenerational, racially mixed pedigree for phenotyping and genotyping for subsequent analyses.

The CARRIAGE (CARolinas Region Interaction of Aging, Genes and Environment) family study is a prospective family-based longitudinal study of the interactions between aging, genetic susceptibility, and environmental risk on the development of several age-related and chronic diseases including OA, cardiovascular disease, and eye disease (glaucoma, and macular degeneration). The extended family described here consists of 3357 pedigreed members dating back nine generations in the United States. Pedigree data were obtained from three sources: 1) a book detailing the genealogy of the descendants of this forefather; 2) family history questionnaires distributed by mail and completed during the family reunions; and 3) genealogy data collected by a family member. These data were combined using Progeny© software for genetic database and pedigree management. We were able to successfully document 3327 family members from the nine generations, with 2795 family members completely connected to the original founder.

Specific Aim 2. Measurement and analysis of OA biomarkers in all sampled family members of a large, multigenerational family and evaluation of their association with OA phenotypes.

In the health fair type setting of our family ascertainment, we could identify OA cases on the basis of clinical examination but not radiography. However, the availability of OA-related biomarkers offers the prospect of powerful tools with which to quantify OA burden. Prior to their use as quantitative traits of OA, we therefore chose to evaluate seven OA biomarkers to understand their relationship to clinical OA in this particular family. To our knowledge, this is the first evidence for an association of OA biomarkers and hand OA based on physical examination alone.

Specific Aim 3. Evaluation of joint hypermobility and its association with OA phenotype and OA biomarkers.

Few studies have evaluated the association between generalized articular hypermobility and osteoarthritis (OA). We evaluated this association and the association of hypermobility and serum concentrations of joint tissue turnover markers, cartilage oligomeric matrix protein (COMP) and hyaluronan (HA). To our knowledge, this is the first study to show an association between joint hypermobility (symptoms-free predominant) and biomarkers.

Specific Aim 4. Determine heritability of OA biomarkers, and joint hypermobility score in a large multigenerational family.

We performed nonparametric variance components analysis (SOLAR[®]) to evaluate heritability for quantitative traits for osteoarthritis indicators (serum HA,

COMP, PIIANP, CPII, C₂C, hs-CRP and GSP), and articular hypermobility (using Beighton score as a continuous covariate).

Specific Aim 5. To use OA biomarkers and joint hypermobility scores as quantitative traits for performing a genome-wide linkage analysis in this family to identify OA susceptibility and OA protective genes/alleles.

We performed a genome-wide linkage analysis in this large, extended pedigree. To define genetic loci for osteoarthritis and joint hypermobility, we used quantitative trait analysis based upon biomarkers with high heritability (serum HA, COMP, PIIANP, C₂C). Quantitative trait analysis also is used to define genetic loci for articular hypermobility based on Beighton scores. To our knowledge, this is first evidence of genetic susceptibility loci identified by OA-related biomarkers in an extended family.

CHAPTER 2

Biomarkers Associated with Clinical Phenotypes of Hand Osteoarthritis in a Large Multigenerational Family: the CARRIAGE Family Study

Reprinted from *Osteoarthritis and Cartilage*. Chen HC, Shah SH, Stabler TV, Li YJ, Kraus VB. 2008;16 (9):1054-9, with permission from Elsevier

2.1 Abstract

Objective: To evaluate biological markers as potential quantitative traits of clinical osteoarthritis (OA) in a large multigenerational family in the Carolinas of the U.S. known as the CARRIAGE family.

Methods: During a series of three family reunions over 6 years, we ascertained 365 family members. We performed clinical hand examinations (n=287), and obtained sera (n=278) for seven OA-related biomarkers (PIIANP, CPII, C₂C, COMP, HA, hs-CRP and glycated serum protein). Three hand OA definitions were evaluated - clinical ACR and GOGO criteria, and any single hand joint involvement. Non-hand OA was defined as a negative hand examination for OA

but varying prevalence of joint symptoms; the control group was defined as having neither symptoms nor evidence for clinical hand OA.

Results: Mean ln HA, ln COMP, and ln hs-CRP were significantly higher in the hand OA group, compared with the non-hand OA or control group. Adjusted for age and sex, mean ln PIIANP (a collagen II synthesis marker) was significantly lower in the hand OA group compared with the other groups. Among those without clinical hand OA, Glycated serum protein was associated with hand joint symptoms.

Conclusions: This is the first report, to our knowledge, showing an association of OA biomarkers and hand OA based on physical examination alone. Analyses using these biomarkers as quantitative traits could reveal novel genetic loci and facilitate exploration of the genetic susceptibility to OA.

2.2 Introduction

Osteoarthritis (OA) is the most common joint disorder causing chronic disability in the United States and worldwide (Lawrence, Helmick et al. 1998; Hogue and Mersfelder 2002). By the year 2030, an estimated 22% of Americans will be affected by arthritis (Elders 2000). OA is regarded as a multifactorial disorder with both environmental and genetic components (Peach, Carr et al. 2005); however, the exact pathogenesis remains unknown. Recently, the genetic

contribution to OA has been increasingly recognized and studied. To date, through whole genome-wide linkage scans, approximately 23 different loci in 14 chromosomes have been reported to link to various OA phenotypes (Jordan, Kraus et al. 2004; Peach, Carr et al. 2005). These studies used a variety of designs, including evaluation of small families of affected relatives (Chapman, Mustafa et al. 1999; Leppavuori, Kujala et al. 1999; Loughlin, Mustafa et al. 1999; Demissie, Cupples et al. 2002; Stefansson, Jonsson et al. 2003; Hunter, Demissie et al. 2004; Greig, Spreckley et al. 2006), twins (Livshits, Kato et al. 2007), and sib-pairs (Kraus, Jordan et al. 2007). So far, many of these genetic loci have been specific to particular populations, suggesting that phenotypic and ethnic variability may complicate the identification of OA susceptibility genes. Considering the challenge of the phenotypic heterogeneity of OA, we exploited a highly unique extended family-based design. The CARRIAGE family is one of the most extensively pedigreed existing families in the United States comprising nine generations originating from one founder born in the 1700's in North Carolina. The ethnic origin of this family is primarily African and Native American. We posited that linkage analysis in this relatively homogeneous large family would provide greater power to identify OA susceptibility genes.

One of the goals of the CARRIAGE family study is to identify novel genes or replicate known genes associated with OA susceptibility and progression. In the health fair type setting of our family ascertainment, we could identify cases on the

basis of clinical examination but not radiograph. However, the availability of OA-related biomarkers offers the prospect of powerful tools with which to quantify OA burden. We therefore chose to evaluate seven OA biomarkers to understand, prior to their use as quantitative traits of OA, their relationship to clinical OA in this particular family. Seven biomarkers were investigated in this large multigenerational family including: HA (hyaluronan), COMP (cartilage oligomeric matrix protein), PIIANP (type IIA collagen N-propeptide), CPII (type II procollagen carboxy-propeptide), C₂C (neoepitope from cleavage of CII), hs-CRP (high-sensitive C-reactive protein) and GSP (glycated serum protein). Several of these markers already fulfilled OA-related criteria for two or more categories of the BIPED (Burden of disease, Investigational, Prognostic, Efficacy of therapy, and Diagnostic) classification scheme (Bauer, Hunter et al. 2006) as follows: these markers match the following categories: HA – B, P; COMP – D, B, P; PIIANP – B, P, D; CPII – P, E, D; C₂C – P, E, D) (Charni-Ben Tabassi and Garnero 2007; Rousseau and Delmas 2007). Although six of the seven biomarkers chosen for this study have been validated previously against radiographic OA criteria, no previous study attempted to estimate the association between these biomarkers and clinical OA criteria. To our knowledge, this is the first evidence for an association of OA biomarkers and hand OA based on physical examination alone.

2.3 Methods

2.3.1 Study population

The CARRIAGE (CARolinas Region Interaction of Aging, Genes and Environment) family study is a prospective family-based longitudinal study of the interactions between aging, genetic susceptibility, and environmental risk on the development of several age-related and chronic diseases including OA, cardiovascular disease, and eye disease (glaucoma, and macular degeneration). The extended family described here consists of 3357 pedigreed members dating back nine generations in the United States. Pedigree data were obtained from three sources: 1) a book detailing the genealogy of the descendants of this forefather; 2) family history questionnaires distributed by mail and completed during the family reunions; and 3) genealogy data collected by a family member. These data were combined using Progeny© software for genetic database and pedigree management. We were able to successfully document 3327 family members from the nine generations, with 2795 family members completely connected to the original founder.

2.3.2 Clinical Ascertainment

Ascertainment of 365 members, 18 years of age and older, was accomplished at three family reunions between 2002 and 2006 and included

blood sampling (n=350), ascertainment of extensive general medical history (n=365), measurement of body mass index (n=309), self-report of joint symptoms (pain, aching, stiffness most days) of the hands, knees, hips, spine, ankles, big toe, shoulders, elbows, and wrists (n=341), and Rheumatologist-performed hand physical examinations for OA (n=278). Two participants were found to have credible evidence for a diagnosis for rheumatoid arthritis and were excluded from these analyses. Additional objective measures collected included: anthropometric data (weight, height), blood pressure, calcaneal bone mineral density using a Norland Apollo™ DEXA, ophthalmologic slit lamp examinations, and physical function testing in members 65 years of age and over. Written informed consent was obtained from each participant and the study was conducted with the approval of the Duke Institutional Review Board. All information and work was conducted under a Federal Certificate of Confidentiality to ensure the privacy of each participating member's clinical and genetic data.

2.3.3 Definitions of hand OA

Hand OA was defined according to three definitions: ACR (American College of Rheumatology) criteria (Altman, Fries et al. 1987), GOGO (Genetics of Generalized OA) criteria (Kraus, Jordan et al. 2007), and any single hand joint involvement. We used modified ACR criteria consisting of 1) hard tissue enlargement of two or more of 10 selected joints; 2) hard tissue enlargement of

two or more DIP joints; and 3) fewer than three swollen metacarpophalangeal (MCP) joints; of note, hand symptoms were not required. The GOGO criteria for determining hand OA affection status consisted of 1) a minimum of 3 joint bony enlargement (of distal or proximal interphalangeal joints - DIP and PIP) or carpometacarpal (CMC-1) squaring, and two of the three joints involving the same joint group; 2) bony enlargement of at least one DIP of digits 2-5; 3) bilateral hand involvement; 4) no more than three swollen MCPs. Any single joint involvement was defined as hard tissue enlargement of any DIP or PIP or CMC-1 squaring.

Participant group status was assigned on the basis of clinical hand examination as either hand OA or non-hand OA. The non-hand OA group had symptoms (pain, aching or stiffness on most days in the last year) in at least one joint system in the body; the control group had neither clinical hand OA nor symptoms in any joint system.

2.3.4 Serum Biomarker Analyses

Blood samples were processed, aliquoted, and stored within 4 hours of collection at -80°C until biomarker analyses were performed. Serum biomarker analyses were repeated as necessary for samples with a $> 15\%$ coefficient of variation (CV).

2.3.4.1 COMP (cartilage oligomeric matrix protein)

COMP was measured by an in-house ELISA method as previously described (Vilim, Olejarova et al. 2002; Elliott, Kraus et al. 2005), using monoclonal antibodies 17C10 and 16F12 against human COMP. The minimum detection limit is 120 ng/ml. Intra-assay and inter-assay CVs were < 5.8% and 8.7%, respectively.

2.3.4.2 HA (hyaluronan)

HA was measured by enzyme-linked binding protein assay (Corgenix Inc. Westminster, Colorado, USA). The assay uses enzyme-conjugated hyaluronic acid binding protein (HABP) from bovine cartilage to specifically capture HA from human serum. The minimum detection limit is established at 10 ng/ml. Intra-assay and inter-assay CVs were < 4.7% and 7.0%, respectively.

2.3.4.3 PIIANP (type IIA collagen N-propeptide)

PIIANP, a marker of a fetal form of collagen II recapitulated in OA, was measured by competitive ELISA (LINCO Research, St. Charles, MO, USA). The minimum

detection limit is 17.2 ng/ml. Intra-assay and inter-assay CVs were < 6.6% and 7.8%, respectively.

2.3.4.4 CPII (type II procollagen carboxy-propeptide)

CPII, a marker of the adult form of collagen II synthesis, was measured by competitive ELISA (IBEX, Montreal, Quebec, Canada). The minimum detection limit is estimated to be 35.1 ng/ml. Intra-assay and inter-assay CVs were < 3.7% and 9.1%, respectively.

2.3.4.5 C₂C (neoepitope from cleavage of CII)

A competitive ELISA (IBEX) was used to measure the neoepitope produced by the cleavage of type II collagen (C₂C). The minimum detection limit is reported as 7.3 ng/ml. Intra-assay and inter-assay CVs are < 2.4% and 9.5%, respectively.

2.3.4.6 Hs-CRP (High-sensitivity C-reactive protein)

Hs-CRP was detected by a solid-phase sandwich ELISA (MAGIWEL; UBI, Mountain View, CA). The minimum detection limit is estimated to be 0.35 ng/ml. Intra-assay and inter-assay CVs were < 3.9% and 8.5%, respectively.

2.3.4.7 GSP (glycated serum protein)

We quantified GSP to measure non-enzymatic glycation. In contrast to hemoglobin A1c (HbA1c), which requires fresh blood for analysis, GSP can be measured in frozen sera. GSP was measured by a specific enzymatic method (DIAZYME) based on direct assessment of fructosamine in serum (Wang, Dou et al. 2005) with colorimetric detection. An assay sensitivity of 30 μ mole/L is reported by the manufacturer. Intra-assay and inter-assay CVs were < 2% and <3%, respectively.

2.4 Statistical Analysis

Biomarker data were natural logarithm transformed to meet assumptions of normal distribution of the data for parametric statistical analysis, performed using GraphPad Prism (GraphPad software, San Diego, CA) and JMP (SAS, Cary, NC) software. Results were analyzed using One-way ANOVA with Tukey-Kramer multiple comparison post-hoc test. Pearson correlation was performed to evaluate for correlation among the biomarkers.

2.5 Results

All ten of the major family branches were sampled and included both descendants of the original founder and married-in members of the family. The baseline characteristics for the 341 participants with available joint symptom data are shown in Table 2.1. Two-thirds of the participants were women. Age and body mass index (BMI) were similar for women and men as were subjective symptoms of the hand joints. Subjective joint complaints were only dissimilar by sex for the spine and big toe.

2.5.1 Biomarkers and Hand Phenotypes

Six of the seven serum biomarkers analyzed were chosen on the basis of literature evidence suggesting some association with OA. To avoid potential confounding by high cartilage turnover due to other arthropathies or by cartilage growth plate metabolism, we excluded 2 participants with rheumatoid arthritis and the 5 participants younger than 25 years of age. Seven serum biomarkers were analyzed for the 271 skeletally mature participants with available hand examination data and sera. Mean In serum HA, COMP, and C₂C were consistently higher in the hand OA group than the non-hand OA and control groups for all definitions of hand OA (Table 2.2).

This difference was significant for HA (all definitions), hs-CRP (all definitions) and COMP (any single hand joint involvement definition). In addition, hs-CRP

was significantly higher in Hand OA and Non-Hand OA (with joint symptoms) than Controls, with the strength of the association varying by hand OA definition.

Overall, five of the seven biomarkers increased significantly with age: HA ($r=0.57$, $p<0.0001$), COMP ($r=0.33$, $p<0.0001$), PIIANP ($r=0.20$, $p=0.0011$), C₂C ($r=0.16$, $p=0.0016$), and hs-CRP ($r=0.12$, $p=0.048$). When adjusted for age and sex, the collagen synthesis marker, PIIANP, was significantly lower in the Hand OA group compared with the Control group for all definitions of hand OA. After age and gender adjustment, the difference in HA among groups was marginally significant (ANOVA $p=0.08$). The age and gender adjusted ratio of HA/PIIANP reflected a significant excess of cartilage degradation over synthesis in the Hand OA group and Non-hand OA (with joint symptoms) group compared with Control (Table 2.3).

Table 2.1 Demographic characteristic of the 341 participants with joint symptom data

Characteristic	Female (n=229)	Male (n=112)
Age, mean \pm SD (range), years	54.87 \pm 15.69 (18~92)	53.31 \pm 15.14 (17~85)
BMI, mean \pm SD (range), kg/m ²	30.60 \pm 6.69 (18.80~60.30) (n=205)	30.90 \pm 5.98 (19.90~52.00) (n=104)
Subjective joint complaints		
Hand symptoms	36 (15.7%)	15 (13.4%)
Knee symptoms	69 (30.1%)	42 (37.5%)
Hip symptoms	39 (17.0%)	12 (10.7%)
Spine symptoms	98 (42.8%)	32 (28.6%)
Ankle symptoms	35 (15.3%)	12 (10.7%)
Shoulder symptoms	66 (28.8%)	23 (20.5%)
Elbow symptoms	20 (8.7%)	7 (6.3%)
Big toe symptoms	18 (7.9%)	1 (0.9%)

Table 2.2 Hand OA and biomarkers (unadjusted)

Modified hand	<i>n</i> = 36	<i>n</i> = 190	<i>n</i> = 45
ACR criteria			
In HA	4.18 ± 0.87 ^{***+}	3.57 ± 0.89 ^{**}	3.07 ± 1.04
In COMP	7.51 ± 0.41 [*]	7.43 ± 0.45	7.30 ± 0.48
In PIIANP	7.20 ± 0.54	7.10 ± 0.50 [*]	7.29 ± 0.53
In CPII	7.17 ± 0.38	7.03 ± 0.41	7.06 ± 0.43
In C ₂ C	5.39 ± 0.22	5.36 ± 0.26	5.35 ± 0.28
In hs-CRP	8.26 ± 1.45	8.28 ± 1.44 ^{**}	7.68 ± 1.16
In GSP	5.44 ± 0.22	5.42 ± 0.25 [*]	5.34 ± 0.21
Age, years	67.7 ± 12.0 ^{***+}	55.9 ± 13.8 ^{**}	48.3 ± 12.8
GOGO hand	<i>n</i> = 41	<i>n</i> = 185	<i>n</i> = 45
criteria			
In HA	4.09 ± 0.86 ^{***+}	3.56 ± 0.90 ^{**}	3.07 ± 1.04
In COMP	7.51 ± 0.38 [*]	7.42 ± 0.45	7.30 ± 0.48
In PIIANP	7.15 ± 0.53	7.10 ± 0.50 [*]	7.29 ± 0.53
In CPII	7.10 ± 0.38	7.04 ± 0.41	7.06 ± 0.43
In C ₂ C	5.40 ± 0.23	5.36 ± 0.26	5.35 ± 0.28
In hs-CRP	8.23 ± 1.44	8.29 ± 1.44 ^{**}	7.68 ± 1.16
In GSP	5.41 ± 0.22	5.43 ± 0.26 [*]	5.34 ± 0.21
Age, years	67.1 ± 12.2 ^{***+}	55.6 ± 13.9 ^{**}	48.3 ± 12.8
Any single hand	<i>n</i> = 108	<i>n</i> = 118	<i>n</i> = 45
joint involvement			
In HA	3.87 ± 0.84 ^{***+}	3.49 ± 0.94 ^{**}	3.07 ± 1.04
In COMP	7.50 ± 0.41 ^{**}	7.38 ± 0.46	7.30 ± 0.48
In PIIANP	7.09 ± 0.50 [*]	7.14 ± 0.52	7.29 ± 0.53
In CPII	7.07 ± 0.43	7.03 ± 0.39	7.06 ± 0.43
In C ₂ C	5.38 ± 0.26	5.35 ± 0.26	5.35 ± 0.28
In hs-CRP	8.32 ± 1.54 ^{**}	8.24 ± 1.35 [*]	7.68 ± 1.16
In GSP	5.43 ± 0.25	5.43 ± 0.24 [*]	5.34 ± 0.21
Age, years	63.7 ± 12.1 ^{***+}	52.3 ± 13.9 ^{**}	48.3 ± 12.8

Values are mean ± SD, ^{*}*P* < 0.1 and ^{**}*P* < 0.05 comparing hand OA or non-hand OA with controls; ⁺⁺*P* < 0.05 comparing hand OA and non-hand OA.

Table 2.3 Hand OA and biomarkers (mean values adjusted for age and/or gender)

	Hand OA	Non-hand OA	Controls	<i>P</i> value (adjusted for age)	Hand OA	Non-hand OA	Controls	<i>P</i> value (adjusted for age and gender)
Modified hand ACR criteria	<i>n</i> =36	<i>n</i> =190	<i>n</i> =45		<i>n</i> =36	<i>n</i> =190	<i>n</i> =45	
ln HA	3.77 ± 0.82	3.58 ± 0.78	3.36 ± 0.80	0.08	3.79 ± 0.84	3.60 ± 0.89	3.37 ± 0.84	0.08
ln PIIANP	7.10 ± 0.52	7.10 ± 0.50	7.36 ± 0.51	0.01	7.04 ± 0.54	7.06 ± 0.56	7.31 ± 0.53	0.01
ln HA/PIIANP	0.53 ± 0.12	0.50 ± 0.11	0.45 ± 0.12	0.01	0.53 ± 0.12	0.51 ± 0.13	0.45 ± 0.12	0.01
GOGO hand criteria	<i>n</i> =41	<i>n</i> =185	<i>n</i> =45		<i>n</i> =41	<i>n</i> =185	<i>n</i> =45	
ln HA	3.70 ± 0.82	3.59 ± 0.77	3.36 ± 0.80	0.1	3.71 ± 0.86	3.61 ± 0.88	3.38 ± 0.84	0.1
ln PIIANP	7.04 ± 0.52	7.11 ± 0.49	7.36 ± 0.51	0.01	6.99 ± 0.54	7.07 ± 0.56	7.31 ± 0.53	0.01
ln HA/PIIANP	0.52 ± 0.12	0.50 ± 0.11	0.45 ± 0.12	0.02	0.53 ± 0.12	0.51 ± 0.13	0.46 ± 0.12	0.02
Any single hand joint involvement	<i>n</i> =108	<i>n</i> =118	<i>n</i> =45		<i>n</i> =108	<i>n</i> =118	<i>n</i> =45	
ln HA	3.59 ± 0.83	3.63 ± 0.83	3.37 ± 0.81	0.2	3.60 ± 0.90	3.64 ± 0.87	3.39 ± 0.84	0.2
ln PIIANP	7.01 ± 0.53	7.18 ± 0.53	7.37 ± 0.51	0.0006	6.97 ± 0.56	7.14 ± 0.54	7.32 ± 0.53	0.0007
ln HA/PIIANP	0.51 ± 0.12	0.50 ± 0.12	0.46 ± 0.12	0.02	0.52 ± 0.13	0.51 ± 0.12	0.46 ± 0.12	0.02

Values are mean ± SD.

2.5.2 Biomarkers and hand symptoms

A total of 120 participants reported joint symptoms in the hand or in another joint system but did not meet the definition of clinical hand OA based on even the least stringent criterion (any single joint involvement). This group was further subdivided into those with hand symptoms +/- symptoms elsewhere (Group A), and those without hand symptoms but with symptoms outside the hand (Group B). We compared these two groups with Controls (Group C) to discern potential biomarkers of early OA. Ln GSP was significantly higher in those with hand symptoms compared with Controls (Figure 2.1). After adjustment for diabetes history, Ln GSP was significantly higher in those with hand symptoms compared with controls ($p=0.039$). Ln HA, and the ratio of HA to collagen synthesis (HA/PIIANP) was significantly higher in those with symptoms outside the hand (Group B) compared with Controls.

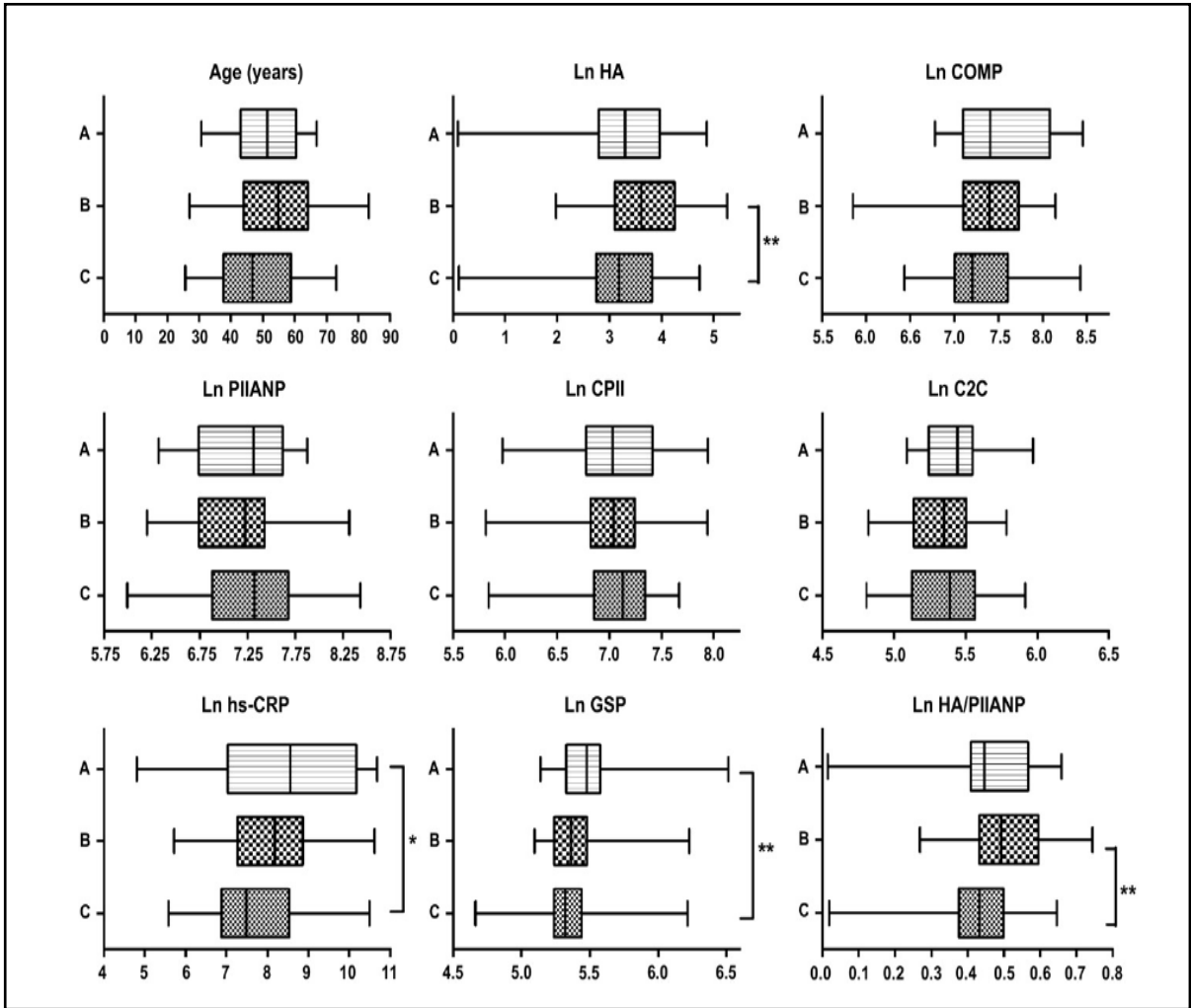


Figure 2.1: Biomarkers and hand symptoms. The individuals without clinical hand OA (on the basis of a negative hand joint examination) were categorized into 3 groups: Group A (n=18) - no hand OA by examination, presence of hand symptoms, and possible symptoms in other joint systems; Group B (n=57) - no

hand OA by examination, no hand symptoms, but joint symptoms in one or more other joint systems; Group C (n=45) - no hand OA by examination, no hand symptoms, and no other joint symptoms. Box plots depict mean, 25th and 75th percentiles, and minimum and maximum. *p<0.1, **p<0.05

2.5.3 Correlations among the Biomarkers

All of the biomarkers, except hs-CRP, correlated with HA (Table 2.4). There was a significant correlation between COMP and glycosylated protein. Not surprisingly, there were strong correlations among the type II collagen biomarkers (PIIANP, CII, and C2C).

Table 2.4 Correlations among the serum biomarkers

Biomarkers	ln HA	ln COMP	ln C2C	ln CII	ln PIIANP	ln hs-CRP
ln COMP	0.3498***					
ln C2C	0.2611***	0.0858				
ln CII	0.2154***	0.0904	0.4434***			
ln PIIANP	0.1976***	0.0685	0.3071***	0.2984***		
ln hs-CRP	0.0819	0.0472	-0.0575	-0.0528	0.0959*	
ln GSP	0.1445**	0.2638***	-0.0344	0.0254	0.0369	0.0454

Correlation coefficients were determined by Pearson correlation; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

2.6 Discussion

We have been able to ascertain multiple members of a large extended family for the purposes of evaluating aging, environmental and genetic risk for OA. Most previous studies of biomarkers and OA were focused on knees and hips and have relied on radiographs. However, compared with knee or hip OA, hand OA is easier to evaluate by physical examination and has been reported to display stronger concordance and familial aggregation. The reunion venues at which these individuals were assessed afforded the possibility of blood collection and limited musculoskeletal examinations, but not radiographic assessment. Based on the strength of past radiographic validation of a number of OA-related biomarkers, we hypothesized that biomarkers could be used as quantitative traits of OA. Our goal therefore, in this study, was to evaluate the strength of association of known OA-related biomarkers to the clinical OA data available in this family.

The ethnic composition of this family is a unique mixture of mainly African and Native American. With a few exceptions (Jordan, Luta et al. 2003; Elliott, Kraus et al. 2005), the majority of OA biomarker validation studies have been performed in Caucasians. This study is therefore novel for specifically evaluating the strength of association between the clinical OA phenotypes and OA-related biomarkers in a non-Caucasian family. This study demonstrated the feasibility of

this goal in that we identified four biomarkers, HA, COMP, PIIANP and GSP, that were associated with hand OA in this family on the basis of clinical examination or hand symptoms. To our knowledge, this is the first report of an association of OA-related biomarkers and hand OA based upon physical examination data alone.

We observed lower PIIANP values in association with hand OA. Although Sharif et al reported that serum PIIANP was higher in knee OA progressors compared with non-progressors, his was a community-based cohort with relatively mild knee OA (Sharif, Kirwan et al. 2007); most of the previous studies have demonstrated that serum PIIANP was decreased in patients with knee OA compared to controls (Garnero, Ayrat et al. 2002; Rousseau, Sandell et al. 2004; Rousseau, Zhu et al. 2004).

We observed an increase in HA and COMP in association with hand OA. In the Johnston County Osteoarthritis biomarkers sub-studies, serum COMP and HA increased significantly with knee and hip OA, even after adjustment for other risk factors including age (Vilim, Olejarova et al. 2002; Elliott, Kraus et al. 2005). The association of serum HA and COMP with hand OA in our cohort was not independent of age. These results may partially arise from the direct comparison of highly sensitive biomarkers with only moderately sensitive physical examination criteria without any supporting radiographs.

Joint symptoms in the absence of clinical examination findings may be an indicator of early OA. We observed higher levels of hs-CRP and GSP in the group with hand symptoms but no hand OA by physical examination. Thus, our data suggest that hs-CRP and GSP may be biomarkers of early sub-clinical hand OA; this possibility has been suggested by several other studies of hs-CRP (Spector, Hart et al. 1997; Sturmer, Brenner et al. 2004). We assessed GSP as an intermediate to advanced glycation end product (AGE) formation. AGEs are formed by non-enzymatic reactions in the process of post-translational modification, have been involved in the aging process and the pathogenesis of several diseases, including rheumatoid arthritis (RA) and diabetes. A recent study has showed accumulation of AGEs is a potential risk factor for OA (DeGroot, Verzijl et al. 2004). Verzijl N et al also showed that AGE crosslinking may result in pathologic stiffness of cartilage *in vitro* (Verzijl, DeGroot et al. 2002). Senolt et al reported an increased serum concentration of pentosidine, a form of AGE, in patients with knee OA. In addition, he found a significant correlation between synovial fluid COMP and serum pentosidine ($R^2=0.11$, $p<0.05$) (Senolt, Braun et al. 2005). In our study, serum glycated protein was correlated with serum COMP ($R^2=0.26$, $p<0.0001$) along with HA ($R^2=0.14$, $p<0.0001$). A larger sample size, or more sensitive phenotyping methods, may be required to see an association between GSP and OA.

A potential limitation of this study was the inability to conduct radiographic phenotyping due to the health fair type setting of the ascertainment venues. We also have knee examination data on only 120 individuals as this aspect of the study was only added in 2006. Therefore, this, as other studies, could be confounded by cartilage degradation at other joint sites for which clinical examination data were unavailable. Nevertheless, we have self-reported arthritis symptoms on all participants for all joint sites. Moreover, our study was community-based and therefore not selected for OA. In this way, it is potentially more representative of the population at large although healthier family members were probably more likely to participate.

In summary, we report the first evidence for an association of OA biomarkers and clinical hand OA. Several of the biological markers (HA, COMP, PIIANP, hs-CRP, GSP, and HA/PIIANP) evaluated in this large family showed an association with clinical phenotypes of hand OA or hand symptoms. This study design offers the prospect of minimizing genetic heterogeneity through the analysis of a large family, and demonstrates the feasibility of utilizing several OA-related biomarkers as quantitative traits to identify underlying OA genes in this family.

CHAPTER 3

Inverse Association of General Joint Hypermobility with Hand and Knee Osteoarthritis and Serum Cartilage Oligomeric Matrix Protein Levels

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3.1 Abstract

Objective: Extensive joint hypermobility, lower serum cartilage oligomeric matrix protein (COMP), and early-onset osteoarthritis (OA) are phenotypes of inherited pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED). However, few studies have evaluated the association between articular hypermobility and primary OA. Therefore, we evaluated this association and tested the hypothesis that COMP level is associated with hypermobility in OA and non-OA individuals.

Methods: Two separate cohorts were available for analysis, the extended CARRIAGE family and a subset of the GOGO sib pair cohort. In the CARRIAGE family, we performed hand and knee examinations, hypermobility evaluations (Beighton criteria), and obtained sera for COMP and hyaluronan (HA). COMP

and HA, extensive joint radiographic and hypermobility data were also available for the GOGO cohort.

Results: The prevalence of hypermobility was 13% in the CARRIAGE and (5%) in the GOGO cohort. In the CARRIAGE family, hypermobility was associated with a significantly lower prevalence of hand (especially proximal interphalangeal joint) and knee OA, and lower mean serum COMP in both the total cohort and non-hand OA subgroups. These results were further validated in the GOGO subsets without radiographic OA where hypermobility was also associated with a significantly lower mean serum COMP ($p < 0.01$). Serum HA did not differ on the basis of hypermobility in either cohort.

Conclusions: We report an inverse relationship of hypermobility, hand and knee OA, and showing that hypermobility is associated with lower serum COMP levels. Genetic variations of the COMP gene may account for some subgroups of benign joint hypermobility.

3.2 Introduction

Osteoarthritis (OA) is a multifactorial complex disorder associated with chronic disability and various risk factors (Jordan, Kraus et al. 2004), including age, obesity, female gender, muscle weakness, joint malalignment, and genetic predisposition (Felson, Lawrence et al. 2000). Joint hypermobility due to ligamentous laxity has empirically been regarded to be a risk factor for OA

(Bridges, Smith et al. 1992), although results of the few studies to date have been conflicting (Table 3.1). The true prevalence and risk of musculoskeletal disorders associated with hypermobility is unknown.

Joint hypermobility, estimated to affect approximately 5% to 25% of the population depending on age, sex, and race (Grahame 1999), is observed as both a lone benign trait and as one manifestation of a variety of severe but rare heritable disorders including Marfan syndrome (1 in 12,000), Ehlers-Danlos syndrome (1 in 5,000), osteogenesis imperfecta (1 in 100,000), pseudoachondroplasia (PSACH, less than 1 in 200,000 in the USA), and some forms of multiple epiphyseal dysplasia (MED, 1 in 10,000) (Briggs and Chapman 2002; Bravo and Wolff 2006). The latter conditions, PSACH and MED, are characterized by prominent joint laxity and variable short stature, short extremities, and early-onset OA, especially in the hips and knees, and are due to mutations in genes coding for cartilage oligomeric matrix protein (COMP) (Briggs, Hoffman et al. 1995). During the ascertainment of the CARRIAGE (CARolinas Region Interaction of Aging Genes and Environment) family, we noticed joint hypermobility in many members. We therefore investigated the association between joint hypermobility and clinical OA phenotypes, and the OA-related serum biomarkers, COMP, and hyaluronan (HA). We then validated our results in a larger family-based study, the GOGO (Genetics of Generalized Osteoarthritis) study.

Table 3.1 Summary of previous studies of OA and articular hypermobility

Author, year (ref.)	Country of origin or ethnic origin of subjects	Study design	No. with hypermobility/total (%)	Hypermobility criteria used	Relationship of joint hypermobility and OA
Scott et al, 1979 (36)	UK	Clinical population; age-matched OA controls	9/100 (9)	Beighton score ≥ 4 and left MCP2 joint involvement	OA increased in general; [†] hand OA decreased; knee OA increased
Bridges et al, 1992 (3)	US	Clinical observation	20/130 (15)	Beighton score ≥ 5	OA in 12 of 20 patients with hypermobility [†]
Jonsson et al, 1995 (34)	Iceland	Patients with established hand OA	19/100 (19)	Beighton score ≥ 4	CMC1 joint OA increased; DIP and PIP joint OA decreased
Jonsson et al, 1996 (35)	Iceland	Female patients with established thumb base OA	17/50 (34)	Beighton score ≥ 4	DIP and PIP joint OA decreased
Dolan et al, 2003 (33)	UK	Postmenopausal female community population	79/716 (11)	Beighton score ≥ 1	Knee OA decreased
Kraus et al, 2004 (32)	Caucasian (UK and US)	OA sibpair family study	39/1,043 (3.7)	Beighton score ≥ 4	PIP joint OA decreased
Present study	Mixed African American and Indian (US)	Extended family-based study	36/280 (13)	Beighton score ≥ 4	PIP joint and knee OA decreased; serum COMP decreased

*MCP2 = second metacarpophalangeal; CMC1 = first carpometacarpal; DIP = distal interphalangeal; PIP = proximal interphalangeal; COMP = cartilage oligomeric matrix protein.

[†] Type of osteoarthritis (OA) not reported.

3.3 Patients and Methods

3.3.1 Study Populations

The CARRIAGE family study is a prospective family-based longitudinal study of the interactions between aging, genetic susceptibility, and environmental risk pertaining to the development of several age-related chronic diseases including OA, cardiovascular disease, and eye diseases (glaucoma, and macular degeneration). This family came to be studied in the context of health fairs we were requested to conduct at several large family reunions. The extended family described here is of mixed African American and native American ancestry and one of the most extensively pedigreed existing families in the United States comprising nine generations with 3357 pedigreed members, and originating from one founder born in the 1700s (Chen, Shah et al. 2008). Ascertainment of 350 family members was conducted during three family reunions from 2002-2006. Ascertainment included physician-performed examinations for hand OA and joint hypermobility (n=287), and knee OA (n=120). Height and weight were measured, and general medical history ascertained including a query or examination for blue sclerae. The majority of these participants (n=278) also consented to blood sampling. For purposes of these analyses, we excluded 2 participants with known clinical rheumatoid arthritis and subjects younger than 25 years of age (n=5) to avoid potential confounding of the biomarker measures by cartilage

growth plate metabolism (Thonar 1990; Urakami, Manki et al. 2006). After these exclusions, a total of 280 participants provided full clinical hand data, a total of 271 had both clinical hand data and biomarker data, and of these, 115 participants also had clinical knee examination data. Written informed consent was obtained from each participant and the study was conducted with the approval of the Duke Institutional Review Board.

The GOGO (Genetics of Generalized Osteoarthritis) cohort is a large sample of Caucasian sibling pairs and nuclear family members ascertained through a collaborative consortium of seven sites in the USA and UK (Kraus, Jordan et al. 2007). Full biomarker data, Beighton scores, and hand, knee, and hip radiographic OA data were available for 708 individuals from two of the sites (Duke University, Durham, NC and University of North Carolina at Chapel Hill, NC, USA). There were no individuals in either the CARRIAGE family or the GOGO cohort with excessively short stature or radiographic features (in the case of the GOGO cohort) suggestive of PSACH or MED, nor was there anyone with blue sclerae.

3.3.2 Survey of joint symptoms in the CARRIAGE family

Information was obtained on self-reported joint symptoms was obtained by asking “Which of the following joints have bothered you in the last year?” The

surveyed joint-sites included hands, knees, hips, spine (neck, upper, lower back), ankles, shoulders, elbows, wrists, and big toes.

3.3.3 Definitions of clinical hand and knee OA outcomes in the CARRIAGE family

OA outcomes in the CARRIAGE family are listed and described in Table 3.2 and included clinical hand OA by modified American College of Rheumatology (ACR) criteria (Altman, Alarcon et al. 1990), clinical hand OA by GOGO criteria (Kraus, Varju et al. 2001; Kraus, Jordan et al. 2007), and clinical knee OA by ACR criteria (Altman, Asch et al. 1986). A Non-Hand OA subgroup in the CARRIAGE family was defined on the basis of not meeting the clinical modified hand OA ACR criteria or the clinical hand OA GOGO criteria.

3.3.4 Definitions of non-OA subgroups in the GOGO cohort

The non-OA subgroups were defined using the available clinical data for the hand as well as on the basis of Kellgren Lawrence (Kellgren and Lawrence 1957) grade <2 radiographic OA of the hands, knees or hips (Table 3.2).

3.3.5 Beighton criteria for hypermobility

Hypermobility was determined according to the criteria established by Beighton et al in 1973 (Beighton, Solomon et al. 1973) that have high inter- and

intra-rater reliability (Boyle, Witt et al. 2003). Patients were graded on a 0-9 point scale based on their ability to achieve the following: (a) passive dorsiflexion of the fifth finger $\geq 90^{\circ}$; (b) passive apposition of the thumb to the forearm; (c) hyperextension of the elbow $\geq 10^{\circ}$; (d) hyperextension of the knee $\geq 10^{\circ}$; and e) ability to rest the palms flat on the floor with straight knees. Beighton scores were analyzed as continuous traits and as binary traits; for binary trait analyses, patients were considered as exhibiting hypermobility if they scored 4 or more out of 9 points (Beighton, Solomon et al. 1973).

Table 3.2 Summary of investigations and definitions

Outcome	Definition	No. with outcome	
		CARRIAGE family (n = 280)	GOGO cohort (n = 708)
Hand OA (modified ACR criteria)	1) Hard tissue enlargement of ≥ 2 of 10 selected joints; 2) hard tissue enlargement of ≥ 2 DIP joints; 3) < 3 swollen MCP joints	47	ND
Hand OA (GOGO criteria)	1) ≥ 3 joints with bone enlargement (of DIP or PIP joints) or CMCI joint squaring, and 2 of the 3 joints involving the same joint group; 2) bone enlargement of ≥ 1 DIP joint of digits 2-5; 3) bilateral hand involvement; 4) ≤ 3 swollen MCP joints Lacks hand OA by modified ACR criteria	52	ND
No hand OA (modified ACR criteria)	Lacks hand OA by GOGO criteria	233	167
No hand OA (GOGO criteria)	Lacks hand OA by GOGO criteria	228	ND
No DIP OA/no PIP OA/ no CMCI OA	Lacks radiographic OA (K/L grade < 2 bilaterally in all joints of the given group)	ND	77/135/333
No knee OA/no hip OA/ no knee or hip OA	Lacks radiographic OA (K/L grade < 2 in joint group bilaterally)	ND	374/399/251
Knee OA (ACR clinical criteria)	Knee pain and a minimum of 3 of 6 other features: 1) age > 50 years, 2) morning stiffness < 30 minutes, 3) crepitus, 4) bone tenderness, 5) bone enlargement, 6) absence of palpable warmth	115	ND
Hypermobility evaluation	Beighton criteria treated as a continuous variable (0-9) or as a dichotomous variable (non-hypermobility < 4 ; hypermobility ≥ 4)	280	708
Serum COMP and HA	Measured by ELISA or binding protein assay	271	708

* For this study, the American College of Rheumatology (ACR) criteria for hand OA were modified in that symptoms and deformity were not included. CARRIAGE = Carolinas Region Interaction of Aging Genes and Environment; GOGO = Genetics of Generalized Osteoarthritis; ND = not determined; K/L = Kellgren/Lawrence; HA = hyaluronan; ELISA = enzyme-linked immunosorbent assay (see Table 1 for other definitions).

3.3.6 Biomarker analyses

Serum was isolated, aliquoted and stored within 4 hours of blood collection at -80°C until biomarker analyses were performed. Duplicate serum biomarker assays were performed for each sample, and analyses were repeated as necessary for samples with a $>15\%$ coefficient of variation (CV). COMP was measured by an in-house sandwich ELISA method as previously described, using monoclonal antibodies 17C10 (epitope in the EGF-like domain) and 16F12 (epitope in the NH_2 -terminal domain) against human COMP (Vilim, Olejarova et al. 2002). The minimum detection limit is 120 ng/ml. Intra-assay and inter-assay CVs were $< 5.8\%$ and 8.7% , respectively. HA was measured by an enzyme-linked binding protein assay (Corgenix Inc. Westminster, Colorado, USA). The assay uses enzyme-conjugated hyaluronic acid binding protein (HABP) from bovine cartilage to specifically capture HA from human serum. The minimum detection limit is established at 10 ng/ml. Intra-assay and inter-assay CVs were $<4.7\%$ and 7.0% , respectively.

3.4 Statistical analyses

The Chi-square test was used to compare the prevalence of joint symptoms and prevalence of OA by hypermobility status. The Mann-Whitney U test was used to assess the mean numbers of OA joints according to hypermobility status. The serum biomarker concentrations were logarithmically transformed to meet

requirements of normality for parametric statistical analyses. Two-sample t test was used to evaluate for differences in mean biomarker concentrations between the hypermobility and non-hypermobility groups. One-Way ANOVA with the Tukey-Kramer multiple comparison test was used to evaluate the relationship of Beighton scores with concentrations of biomarkers. Generalized Estimating Equations (GEE) were used to control for the dependency due to familial clustering of CARRIAGE family members and GOGO nuclear families (SAS version 9.1, SAS Institute, Cary, NC). For the CARRIAGE family, we classified individuals into eight clusters based on their relationship to eight members descended from the founder of the CARRIAGE family. For the GOGO cohort, individuals were clustered by families. Age adjustment was performed for all analyses and additional adjustment for BMI was performed for all analyses involving prevalence of clinical or radiographic OA. Adjustment for hand OA status was included in the logistic regression analysis of hypermobility and COMP in the CARRIAGE family. Analyses of hypermobility and COMP were performed in the full GOGO sample of patients from two sites (Duke and UNC) and in subgroups without radiographic OA. Significant results were declared based on a two-sided p-value of <0.05.

3.5 Results

3.5.1 Hypermobility and joint symptoms in the CARRIAGE family

Joint hypermobility (Beighton score ≥ 4) was present in 36 (12.9%) of the 280 examined CARRIAGE family participants. The hypermobility group did not differ significantly by age or BMI from the non-hypermobility group (Table 3). The age distribution of the 115 participants in the knee-examined subgroup was similar to the group as a whole with a slightly younger mean age for the hypermobility group, which was not statistically different from the non-hypermobility group (53.5 ± 14.2 years for hypermobile individuals, 58.3 ± 14.7 years for non-hypermobile individuals, $p=0.15$). The hypermobility group had a female predominance compared with the non-hypermobile group. The prevalence of hand and knee joint symptoms was lower in the hypermobile group, but the prevalence of symptoms in the other joints systems was similar (Table 3.3).

Table 3.3 Demographic characteristics of the CARRIAGE family study participants

	Hypermobility group (Beighton score ≥ 4) (n = 36)	Non-hypermobility group (Beighton score < 4) (n = 244)	P†
Age, mean \pm SD years	53.27 \pm 14.38	56.36 \pm 14.64	0.24
BMI, mean \pm SD kg/m ²	29.67 \pm 6.09	31.23 \pm 6.67	0.19
Female	30 (83.3)	162 (66.4)	0.03
Self-reported joint symptoms			
Hand	1 (4.0)	47 (21.0)	0.02
Knee	5 (20.0)	92 (41.1)	0.03
Hip	5 (20.0)	43 (19.2)	0.92
Spine	12 (48.0)	101 (45.1)	0.78
Ankle	4 (16.0)	38 (17.0)	0.90
Shoulder	7 (28.0)	68 (30.4)	0.80
Elbow	2 (8.0)	23 (10.3)	0.71
Wrist	3 (12.0)	30 (13.4)	0.84
Big toe	2 (8)	16 (7.1)	0.87

* Except where indicated otherwise, values are the number (%) (based on a total group of 25 in the hypermobility group and 224 in the non-hypermobility group with available joint symptom data).

† By unpaired *t*-test for evaluation of age and body mass index (BMI); by likelihood ratio chi-square test for evaluation of self-reported joint symptoms.

3.5.2 Hypermobility and Osteoarthritis in the CARRIAGE family

The hypermobility group in the CARRIAGE family had a consistently lower prevalence of hand OA by the modified ACR and GOGO criteria, and a lower prevalence of knee OA by ACR criteria (Table 3.4). By logistic regression, hypermobility was associated with a decreased likelihood ratio of hand OA in the CARRIAGE family ($p=0.02$ by modified ACR criteria; $p=0.008$ by GOGO criteria), which remained significant after age and BMI adjustment (for modified ACR criteria: $p=0.024$ BMI-adjusted, $p=0.043$ age-adjusted, and $p=0.047$ age and BMI adjusted; for GOGO criteria: $p=0.009$ BMI-adjusted, $p=0.018$ age-adjusted, and $p=0.02$ age and BMI adjusted) (Table 3.4). The hypermobility group demonstrated significantly fewer OA clinically affected PIP joints ($p<0.005$), and a non-significant but decreased prevalence of OA of DIP and CMC₁ joints. Taking all three hand joint groups into consideration, hypermobility was significantly inversely associated with numbers of OA affected joints (Table 3.4). Hypermobility was also associated with a decreased likelihood ratio of knee OA ($p=0.02$; $p=0.035$ BMI-adjusted, $p=0.058$ age-adjusted, and $p=0.068$ age and BMI adjusted).

Table 3.4 Clinical OA status by hypermobility status in the CARRIAGE family

OA definition†	Hypermobility group (Beighton score ≥4)	Non-hypermobility group (Beighton score <4)	P‡	Age- and BMI- adjusted P‡
Hand (GOGO criteria) (n = 280)				
GOGO criterion 1	1 (2.8)	42 (17.2)	0.008	0.02
GOGO criterion 2	1 (2.8)	57 (23.4)	<0.001	<0.01
GOGO criterion 3	5 (13.9)	60 (24.6)	0.13	0.3
GOGO criterion 4	4 (11.1)	71 (29.1)	0.01	0.02
Hand (modified ACR criteria) (n = 280)				
ACR criterion 1	1 (2.8)	36 (14.8)	0.02	0.047
ACR criterion 2	2 (5.6)	68 (27.9)	<0.005	<0.005
Knee (ACR criteria) (n = 115)	2 (5.6)	39 (16.0)	0.06	0.14
Joint site involvement (n = 280), mean ± SD	3 (12.0)	31 (34.4)	0.02	0.068
No. of joints with OA				
DIP joints	0.22 ± 0.63	0.60 ± 1.35	0.13	0.18
PIP joints	0.14 ± 0.49	1.01 ± 1.97	<0.005	0.01
CMC1 joints	0.028 ± 0.17	0.14 ± 0.48	0.2	0.24
DIP + PIP + CMC1 joints	0.39 ± 0.99	1.75 ± 3.12	<0.005	0.02

* In the studies of hand OA, n = 36 and 244 in the hypermobility group and the non-hypermobility group, respectively; in the studies of knee OA, n = 25 and 90 in the hypermobility group and the non-hypermobility group, respectively. Except where indicated otherwise, values are the number (%). CARRIAGE = Carolinas Region Interaction of Aging Genes and Environment (see Table 1 for other definitions).

† See Table 2 for description of the modified American College of Rheumatology (ACR) criteria and the Genetics of Generalized Osteoarthritis (GOGO) criteria.

‡ By likelihood ratio chi-square test for OA definition; by Mann-Whitney U test for joint site involvement.

3.5.3 Hypermobility and biomarkers in the CARRIAGE family

Mean (SD) In serum COMP decreased significantly with increasing hypermobility by Beighton score: 7.48 ± 0.42 for Beighton score 0; 7.37 ± 0.42 for Beighton score 1-3; 7.29 ± 0.43 for Beighton score ≥ 4 ($p=0.034$ adjusted for age, Figure 3.1a). Mean (SD) In serum COMP was significantly lower in the hypermobility group (7.29 ± 0.43) vs. the non-hypermobility group (7.43 ± 0.42) ($p=0.05$) (Figure 3.1b); however, mean In serum HA did not differ significantly between the two groups (Figure 3.1c). When adjusted for age and hand OA status (by clinical GOGO or modified hand ACR criteria), mean In serum COMP was marginally significantly lower in the hypermobility group compared with the non-hypermobility group (by GOGO criteria, $p=0.069$; by modified hand ACR criteria, $p=0.068$).

To more clearly define the association of hypermobility and serum COMP, independent of hand OA status, we analyzed the relationship in the Non-Hand OA subgroup of the CARRIAGE family. Mean In serum COMP was also significantly lower in the Non-Hand OA subgroup with hypermobility compared with the members without hypermobility. When hand OA was excluded on the basis of clinical GOGO criteria, mean In serum COMP levels were 7.26 ± 0.43 in the hypermobility group vs 7.42 ± 0.43 in the non-hypermobility group ($p=0.053$ adjusted for age); when hand OA was excluded on the basis of the modified ACR

criteria, mean ln serum COMP was 7.27 ± 0.42 in the hypermobility group vs 7.42 ± 0.41 in the non-hypermobility group ($p=0.049$ adjusted for age) (Figure 1d and 1e). When familial clusters were taken into account by the GEE analysis, the inverse association between, hypermobility (using Beighton score as a continuous covariate) and serum COMP level remained highly significant ($p=0.0035$ age-adjusted). When hypermobility was analyzed as a binary trait (<4 or ≥ 4), the association was still apparent but less robust ($p=0.082$ age-adjusted).

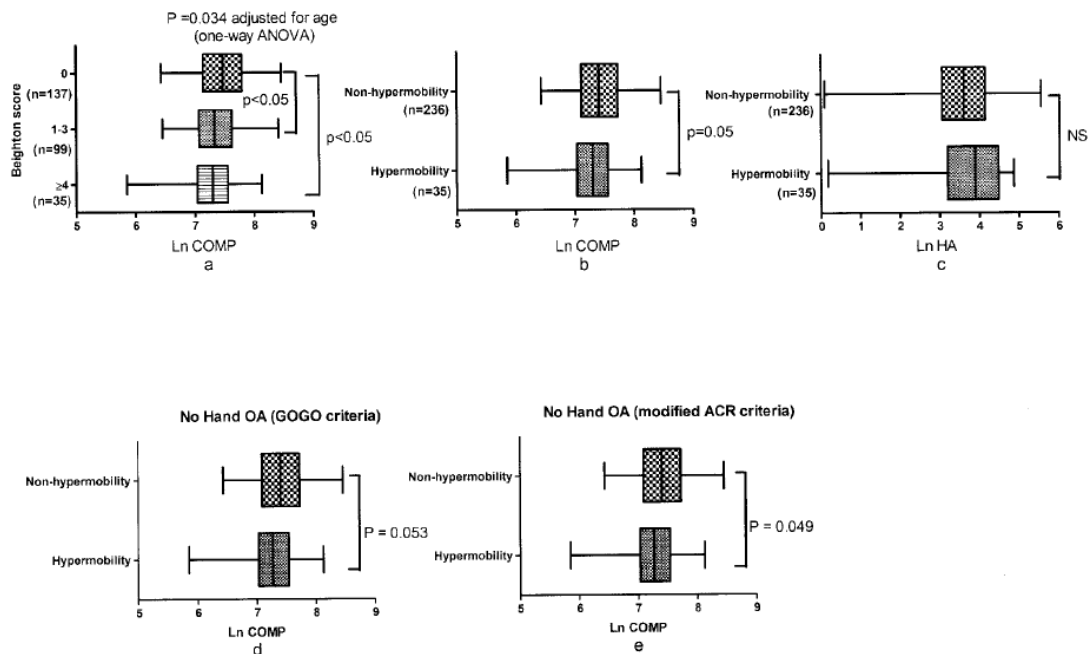


Figure 3.1: The relationship between biomarkers and joint hypermobility in the total cohort (a-c) and Non-Hand OA subgroups (d-e) of the CARRIAGE family. a) Serum Ln COMP (after adjustment for age) and Beighton score separated into 3 groups, no hypermobility (score 0), moderate hypermobility (score 1-3) and high hypermobility (score ≥ 4); b) Serum Ln COMP by hypermobility status; c) Serum Ln HA by hypermobility status. Analysis of participants without hand OA was based on d) clinical GOGO criteria (n=228: n=34 with and n=194 without joint hypermobility); or e) modified ACR criteria (n=233: n=34 with and n=199 without joint hypermobility). P value calculated by one-way ANOVA with Tukey multiple comparison (a), or two-sample t test (b-e). Box plots depict mean, 25th and 75th percentiles, and minimum and maximum.

Hypermobility status defined by Beighton score <4 (Non-hypermobility) or ≥ 4 (Hypermobility). COMP = cartilage oligomeric matrix protein, HA = hyaluronan, NS = non-significant.

3.5.4 Hypermobility and biomarkers in the GOGO cohort

Joint hypermobility was present in 36 (5%) of the 708 GOGO study participants. In agreement with the CARRIAGE family results, mean (\pm SD) ln serum COMP in the GOGO cohort also decreased significantly with increasing hypermobility by Beighton score: 6.94 ± 0.53 for Beighton score 0; 6.72 ± 0.53 for Beighton score 1-3; 6.63 ± 0.54 for Beighton score ≥ 4 ($p < 0.0001$, adjusted for age). Mean ln serum COMP was consistently lower in the hypermobility group compared with the non-hypermobility group (6.60 ± 0.61 vs 6.91 ± 0.53 , $p = 0.0009$; 6.64 ± 0.54 vs 6.90 ± 0.53 , $p = 0.004$ adjusted for age). In contrast, before and after controlling for age, mean ln serum HA was similar in the two groups (3.44 ± 0.88 vs 3.63 ± 0.85 , $p = 0.2$; 3.63 ± 0.81 vs 3.62 ± 0.80 , $p = 0.9$ adjusted for age).

To evaluate the possibility that OA itself might cause an apparent diminution in manifestations of hypermobility due to loss of joint range of motion, we repeated the analyses in the individuals without radiographic OA (rOA). For each joint site, mean ln serum COMP was significantly lower in association with joint hypermobility (Figure 3.2). Results for the 251 individuals lacking both hip and knee rOA were similar, with lower serum COMP in the hypermobile individuals ($p = 0.002$ adjusted for age). In contrast, mean ln serum HA did not differ on the basis of hypermobility status in the non-rOA groups. Similar to the CARRIAGE family dataset, the GEE analysis for GOGO data revealed a highly significant

inverse association between hypermobility (using Beighton score as a continuous covariate) and serum COMP level ($p < 0.0001$ age-adjusted). When hypermobility was analyzed as a binary trait (< 4 or ≥ 4), the inverse association with serum COMP level was also apparent ($p = 0.01$ age-adjusted).

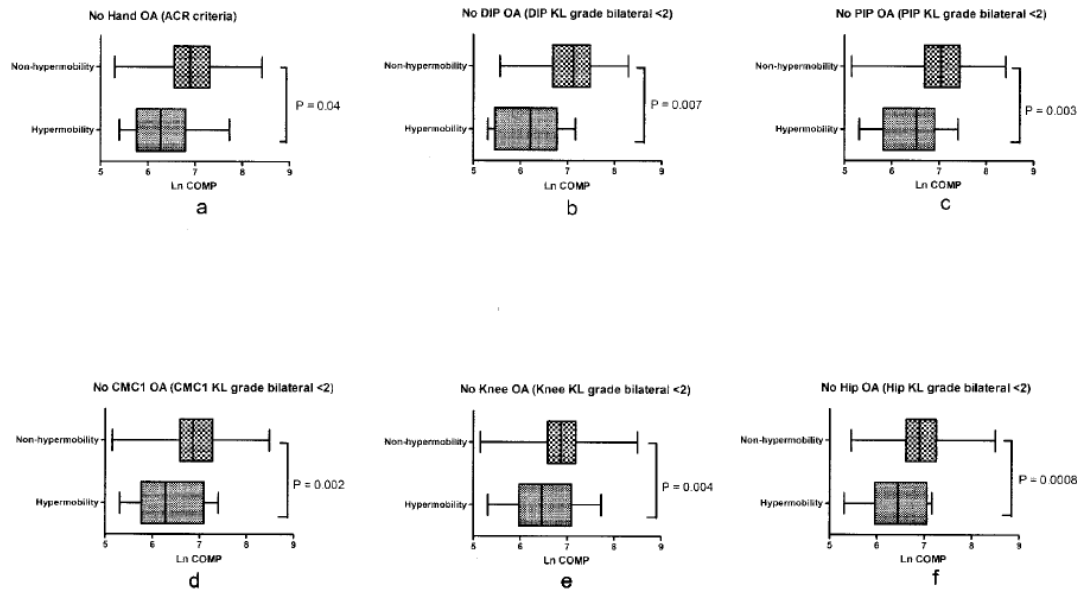


Figure 3.2: Mean Ln COMP by hypermobility status in the Non-OA subgroups of the GOGO cohort. Analysis of participants without OA of the following specific joint groups: a) hand OA based on ACR criteria (n=167); b) rOA of DIP joints (n=77); c) rOA of PIP joints (n=135); d) rOA of CMC₁ joints (n=333); e) rOA of knees (n=374); or f) rOA of hips (n=399). P value (adjusted for age) calculated by two-sample t test with equal variance. Box plots depict mean, 25th and 75th percentiles, and minimum and maximum. Hypermobility status defined by Beighton score: <4 (Non-hypermobility) and ≥4 (Hypermobility). DIP = distal interphalangeal; PIP = proximal interphalangeal; CMC₁ = first carpometacarpal; KL grade = Kellgren-Lawrence grade radiographic OA (rOA); COMP = cartilage oligomeric matrix protein

3.6 Discussion

The present results provide evidence of a relationship between serum COMP levels and general joint hypermobility. COMP is a 524 KDa homopentameric non-collagenous glycoprotein derived from cartilage and also found in ligaments and tendons (Muller, Michel et al. 1998). Recent *in vitro* studies have shown that COMP can interact with collagens I, II, IX, fibronectin, and all matrilins (Rosenberg, Olsson et al. 1998; Holden, Meadows et al. 2001; Di Cesare, Chen et al. 2002; Mann, Ozbek et al. 2004), and that COMP can bind to collagens I, II, and IX with high affinity (Thur, Rosenberg et al. 2001). Interestingly, some autosomal dominant osteochondrodysplasias (PSACH and some MED) are caused by mutations in COMP that interfere with normal extracellular matrix assembly, which is thought to contribute to the development of the patient phenotypes (Chen, Stevens et al. 2004; Schmitz, Becker et al. 2006). Pronounced hypermobility and low serum COMP are features of these osteochondrodysplasias (Mabuchi, Momohara et al. 2004; Tufan, Satiroglu-Tufan et al. 2007).

Low serum COMP may result from retention of mutant COMP within the rough endoplasmic reticulum of chondrocytes and tendon cells (Weirich, Keene et al. 2007); but not all the COMP-associated chondrodysplasias appear to be storage diseases (Chen, Stevens et al. 2004; Schmitz, Becker et al. 2006), so other mechanisms yet to be defined, such as altered COMP protein or RNA

synthesis or stability, may account for low serum COMP in these chondrodysplasias. By analogy, genetic variation within the COMP gene might influence both serum COMP levels and ligamentous structure leading to articular hypermobility phenotypes in the CARRIAGE family and GOGO cohort. Of note, Jonsson has recently reported linkage of joint hypermobility (dorsiflexion $\geq 90^\circ$ of either fifth finger in an Icelandic cohort of 331 subjects) to chromosome 19P 13.3 (LOD score of 3.8), which is within 16Mb of the COMP gene (Jonsson, Ingvarsson et al. 2007). Also, Hakim et al, has reported autosomal dominant inheritance of benign joint hypermobility affecting female twins (Hakim, Cherkas et al. 2004).

Our study also demonstrated that generalized articular hypermobility is inversely associated with clinical hand (PIP) OA and possibly also knee OA. This confirms and extends our previous results in the GOGO cohort showing that hypermobility was associated with a lower prevalence of PIP OA and possibly OA in MCP joints. A strength of this study is that all family members were invited to participate and included, independent of hypermobility status or signs or symptoms of musculoskeletal problems. Although it is possible that the healthier family members may have been more likely to attend the family reunions, we avoided the common selection bias of most other studies related to hypermobility that relied on clinic based populations with a high prevalence of joint symptoms. These family data may therefore be more representative of the general

population. Our study showed that after accounting for age, PIP joint and knee OA prevalence was lower in association with joint hypermobility with a similar trend observed for DIP and CMC₁ joint OA. In the previously reported study of hypermobility in the GOGO cohort, no conclusions could be drawn regarding hypermobility and DIP joints because study inclusion required OA in at least one DIP in the proband and one sibling (Kraus, Li et al. 2004; Kraus, Jordan et al. 2007). No such inclusion criteria were used in the CARRIAGE family study and we saw a trend of fewer OA affected DIP joints in association with hypermobility. It is possible that a larger sample size or radiographic phenotyping might be necessary for further validation of the inverse relationship of hypermobility and OA of DIP, MCP and CMC₁ joints.

Our results are also in agreement with a recent community-based study of post-menopausal females showing a reduced risk of radiographic knee OA with joint hypermobility (Dolan, Hart et al. 2003). Preliminary data from another study, a cohort of Icelandic subjects (n=1839) with a 31% prevalence of any hypermobility, has also shown a reduction in clinical knee OA in association with hypermobility (Chi square $p = 0.04$, Dr. Helgi Jonsson personal communication). Moreover, Jonsson et al reported, in two separate studies, less hand interphalangeal joint involvement in association with hypermobility (Jonsson and Valtysdottir 1995; Jonsson, Valtysdottir et al. 1996). In contrast, some studies have reported a higher prevalence of OA in association with hypermobility.

Decades ago, two studies reported a higher prevalence of OA in individuals with joint hypermobility from groups of highly selected patients referred for clinical evaluation (Scott, Bird et al. 1979; Bridges, Smith et al. 1992); however, in one of these studies, different effects were observed for the knee (increased OA) compared with hand (decreased OA) in association with hypermobility (Scott, Bird et al. 1979). Jonsson et al also reported more CMC₁ joint OA in association with hypermobility (Jonsson and Valtysdottir 1995). Thus, the type and strength of the effect of hypermobility on OA susceptibility may differ by joint group,

Although several previous studies have emphasized the association of the benign joint hypermobility syndrome with musculoskeletal symptoms (Adib, Davies et al. 2005; Ferrell, Tennant et al. 2007; Remvig, Jensen et al. 2007), our study showed that CARRIAGE family members with joint hypermobility had a lower prevalence of self-reported joint symptoms in their hands and knees than participants without hypermobility. Moreover, it has been recognized through studies of children and adolescents, that not all individuals deemed hypermobile have a history of musculoskeletal symptoms and disorders or go on to develop them in their life (Murray 2006). In agreement with our study, Larsson et al also found a lower prevalence of hand symptoms in instrumentalists with lax fingers performing repetitive fine hand movements compared with their peers with less flexibility (Larsson, Baum et al. 1993). The effect of hypermobility on symptoms may be specific to particular joint groups since hypermobility appeared to

increase low back symptoms in timpani players (Larsson, Baum et al. 1993). Thus, again joint group is one possible factor explaining some of the differences in hypermobility related symptoms and OA risk.

There are many other possible factors that might explain the reports of conflicting associations of hypermobility, osteoarthritis and musculoskeletal symptoms. Murray has suggested that hypermobility alone may not account for musculoskeletal syndromes but that other cofactors, such as obesity, sedentary lifestyle, or joint overuse may be important moderators of the symptoms and outcomes of hypermobility (Murray 2006). Murray has also suggested that a high risk subgroup of children may exist which would be under recognized when the typical Beighton score thresholds used for adults are employed for defining hypermobility (Murray 2006). Mechanical joint forces may vary due to ligamentous laxity on a joint specific basis. It is possible that individuals with hypermobility may moderate their activity due to pain or joint instability that may reduce the risk of OA.

Finally, hypermobile individuals represent both a phenotypically and genotypically heterogeneous group. In addition to PSACH and MED (COMP mutations), variable degrees of hypermobility are associated with other genetic syndromes including among others, Marfan's syndrome (fibrillin mutations), and Ehlers Danlos syndromes (mutations of col1A1, col1A2, col3A1, col5A1, col5A2, ADAMTS2, and tenascin XB) (Wikipedia 2008). The underlying genetic etiology

rather than hypermobility, may account for the risk of symptoms and OA, but under diagnosis of these conditions may in part be responsible for the general but erroneous attribution of all hypermobility with risk of OA. These issues deserve to be further explored in future studies.

Although we obtained the same results from two separate cohorts, some shortcomings remain. We are limited due to the cross-sectional nature of our study. Therefore, we cannot completely rule out the possibility that OA masks the manifestations of hypermobility, although OA seldom affects the wrists or 5th MCP joints that contribute to the Beighton score. However, in our study, we believe that the inverse association of hypermobility and OA was not due to waning joint laxity with age and OA because the negative association persisted after adjustment for age. We considered the possibility that lower COMP in the hypermobility group might be a manifestation of younger age and fewer OA affected joints since serum COMP is positively associated with severity of radiographic OA (Vilim, Olejarova et al. 2002; Kelman, Lui et al. 2006). Therefore, we also analyzed the non-OA participants to evaluate the association of hypermobility and the biomarkers, COMP and HA. Serum COMP level was consistently lower in association with hypermobility in both cohorts of non-OA individuals. In contrast, HA levels, a marker of joint tissue turnover of OA (Elliott, Kraus et al. 2005), were unchanged in association with hypermobility. We thus show that the association of hypermobility with lower prevalence of OA and lower

serum COMP, was neither a result of age nor a result of OA masking hypermobility.

To address the possibility that COMP fragmentation was a cause of lower serum COMP with hypermobility, we performed additional sandwich ELISAs, and Western blots (data not shown) on the sera of a test subset of 9 individuals with and without hypermobility, low and high COMP, and OA, using three different monoclonal antibodies to the COMP amino-terminus, middle and carboxy-terminus as described previously (Vilim, Lenz et al. 1997) (reducing gels for antibodies of 16F12 and 12C4; nonreducing gel for antibody of 17C10). Specifically, we evaluated these sera for evidence of COMP fragments between 50-90 kDa as described previously using polyclonal antibodies against human COMP (Neidhart, Hauser et al. 1997). There were no 50-90 kDa COMP fragments in the sera of any of the test individuals, but COMP fragments of this size were readily discerned in the positive control (EDTA cartilage extract). ELISA assays with combinations of these three anti-COMP antibodies also supported the presence of full-length COMP in the sera (Figure 3.3). Thus, the lower serum COMP level associated with hypermobility was due to absolute lower level of full-length COMP in the sera rather than targeted degradation.

COMP Western blots

H=Hypermobility patient sera
NH=non-Hypermobility pateint sera

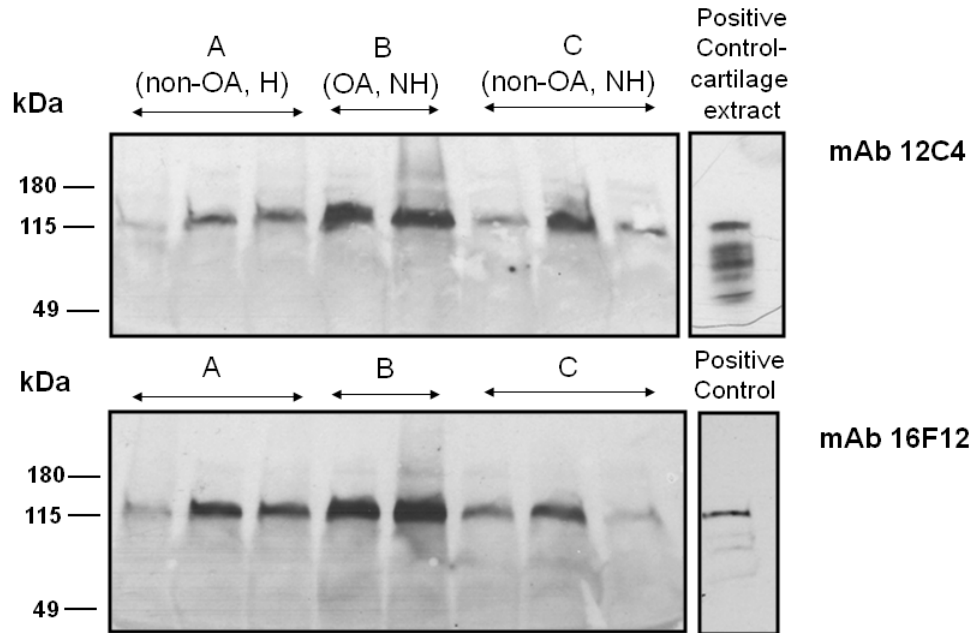


Figure 3.3: Western blots of serum COMP. Serum COMP of 9 individuals was assessed by Western blot under reducing conditions that would release fragments. We analyzed n=3 hypermobile individuals with the lowest COMP levels for their group; n=2 non-hypermobile individuals with the highest COMP levels for their group; and n=3 non-hypermobile individuals with the lowest COMP levels for their group. There were no COMP fragments in the serum that were discernible by Western blots using the antibodies to either the N- or C-terminus of COMP. The positive control (cartilage extract) showed the typical

ladder of COMP fragments seen in cartilage or synovial fluid as described previously by Vilim (Archives of Biochemistry & Biophysics 1997;341(1):8-16).

In summary, we report the evidence for a relationship between serum COMP level and general joint hypermobility. In addition, in this extended family of mixed African American and Native American heritage, we have replicated the results from the GOGO Caucasian sib pair cohort, in which we demonstrated that general articular hypermobility was inversely associated with OA of PIP joints. Our study also suggests the possibility of an inverse association of joint hypermobility and knee OA although this result needs to be validated in a larger cohort. We hypothesize that hypermobility may decrease biomechanical strain on joints, which in turn would be protective for hand and knee OA. Hypermobility is a heritable trait associated with lower serum COMP and COMP mutations in some osteochondrodystrophies. The results of our study suggest that genetic variation within the COMP gene may be a candidate to account for benign joint hypermobility, a condition whose etiology has hitherto been unknown. This study also suggests that the extent of hypermobility might serve as a quantitative trait for identifying protective alleles for OA.

CHAPTER 4

Genome-Wide Linkage Analysis of Quantitative Biomarker Traits of Osteoarthritis and Quantitative Articular Hypermobility

4.1 Abstract

Objective: The genetic contributions to the multifactorial disorder Osteoarthritis (OA) have been increasingly recognized. Our goal was to use OA-related biomarkers of severity and disease burden as quantitative traits to identify genetic susceptibility loci for OA.

Methods: In the extended CARRIAGE family (n=350), we measured five OA-related biomarkers: HA (hyaluronan), COMP (cartilage oligomeric matrix protein), PIIANP (type IIA collagen N-propeptide), CPII (type II procollagen carboxy-propeptide), and C₂C (neoepitope from cleavage of Collagen II). A total of 6,090 SNP markers covering the whole genome were genotyped using the Illumina HumanLinkage-12 BeadChip. SOLAR was used to estimate heritabilities of the quantitative traits, and to calculate two-point and multi-point LOD scores using a polygenic model.

Results: Four of the five biomarkers showed significant heritability ($p \leq 0.01$ age and sex adjusted: PIIANP 57%, HA 49%, COMP 43%, C₂C 30%; not significant: CPII 3%). Fourteen of the 19 loci with multi-point LOD scores >1.5 were near or

overlapped previously reported OA susceptibility loci. Four of these loci were identified by more than one biomarker. The maximum multi-point LOD scores for the heritable quantitative biomarker traits were LOD 4.3 for PIIANP (chromosome 8p23); LOD 3.2 for COMP (chromosome 8q11.1); LOD 2.0 for HA (chromosome 6q16); LOD 2.0 for C2C (chromosome 5q31).

Conclusions: We report the first evidence of genetic susceptibility loci identified by OA-related biomarkers in an extended family. We showed that serum concentrations of PIIANP, HA, COMP and C₂C have substantial heritable components and identified several genetic loci potentially contributing to the genetic diversity of OA.

4.2 Introduction

Osteoarthritis (OA) is the most common joint disorder worldwide and the most common cause of disability in Western countries with significant socio-economic consequences (Brooks 2002). Although many studies have shown that OA has a strong genetic component (Jordan, Kraus et al. 2004; Peach, Carr et al. 2005; Ikegawa 2007), with an estimated heritability ranging from 39 to 74% based on the pattern of joint involvement (Valdes and Spector 2008), the genetic and phenotypic heterogeneity of OA presents challenges in the ongoing attempt to identify the genetic contributions to this complex disease (Chanock, Manolio et al. 2007). Over the last decade the whole-genome linkage scan approach has led

to the identification of a number of susceptibility loci for OA. These findings have all relied on phenotyping by radiograph, clinical examination, or clinical history (total joint replacement) (Chapman, Mustafa et al. 1999; Leppavuori, Kujala et al. 1999; Loughlin, Mustafa et al. 1999; Demissie, Cupples et al. 2002; Stefansson, Jonsson et al. 2003; Forster, Chapman et al. 2004; Hunter, Demissie et al. 2004; Loughlin, Dowling et al. 2004; Greig, Spreckley et al. 2006). However, the hallmark of OA is cartilage loss. Reflected on radiographs as the joint-space width, it is a fairly late stage manifestation of disease with poor sensitivity for OA initiation (Garnero 2006). An alternative approach, the use of intermediate biomarker traits, has been used successfully for genetic analyses of other diseases [anti-cyclic citrullinated peptide in rheumatoid arthritis (Plenge, Seielstad et al. 2007) and YKL-40 in asthma (Ober, Tan et al. 2008)], but never in OA. Existing OA-related biomarkers have the potential to detect disease earlier than is possible by radiograph (Bleasel, Poole et al. 1999), and to reflect not only OA severity but also total body burden of disease (Kraus VB 2009). Moreover, OA is clearly not only a cartilage disorder (Quasnicka, Anderson-MacKenzie et al. 2006; Mansell, Collins et al. 2007) but rather a disease of the whole joint organ consisting of cartilage, bone, synovium, meniscus and tendon. We hypothesized that we could identify known OA susceptibility genes and identify additional OA-related genes and shared genetic determinants through monitoring of the turnover of the whole joint organ through a biomarker approach, thereby potentially providing data that could augment existing knowledge of OA etiologic

pathways and progression. Based on the strength of previous validation studies (J Cibere 2009), we chose to analyze five serum OA-related biomarkers in this study: hyaluronan (HA), cartilage oligomeric matrix protein (COMP), type IIA collagen N-propeptide (PIIANP), type II procollagen carboxy-propeptide (CPII), and type II collagen neopeptide (C₂C). Each of these markers has data to support their classification (Charni-Ben Tabassi and Garnero 2007; Rousseau and Delmas 2007) in at least two categories of the BIPED (Bauer, Hunter et al. 2006) biomarker classification scheme (Burden of disease, Investigational, Prognostic, Efficacy of therapy, and Diagnostic markers): HA – categories B, P; COMP – categories D, B, P; PIIANP – categories B, P, D; CPII – categories P, E, D; and C₂C – categories P, E, D.

For these analyses, we studied a unique extended family, the CARRIAGE (CARolinas Region Interaction of Aging, Genes and Environment) family. This extended family is one of the most extensively pedigreed existing families in the United States comprising 10 generations with 3327 pedigreed members, and originating from one founder born in the 1700s (Chen, Shah et al. 2008). The ethnic origin of this family is primarily African and Native American (Chen, Shah et al. 2008). Linkage analysis in this single founder lineage provides many advantages for mapping complex traits due to the reduction of genetic heterogeneity and confounding by population stratification (Ober, Abney et al. 2001). In this cohort, we have previously documented a prevalence of clinical hand OA of 17% and clinical knee OA of 41% based on American College of

Rheumatology criteria (Chen, Shah et al. 2008). We have also observed an association of hand OA phenotypes in this cohort with serum OA-related biomarkers (Chen, Shah et al. 2008). Here, we report the first evidence for genetic linkage in OA using these biomarker traits in this large extended family.

4.3 Methods

4.3.1 Family Cohort

Pedigree data for the CARRIAGE family were obtained from three sources: 1) a book detailing the genealogy of the descendants of this forefather; 2) family history questionnaires distributed by mail and completed during three family reunions over 4 years (2002, 2004 and 2006); and 3) genealogy data collected by a family member. These data were combined using Progeny© software (www.progenygenetics.com) for genetic database and pedigree management. We were able to successfully document 3327 family members from the nine generations, with 2795 family members completely connected to the original founder. This family came to be studied in the context of health fairs conducted at several large family reunions. Detailed ascertainment of 350 family members was accomplished during the three family reunions. Written informed consent was obtained from each participant and the study was approved by the Duke Institutional Review Board. All information and work was conducted under a

Federal Certificate of Confidentiality to ensure the privacy of each participating member's clinical and genetic data.

4.3.2 Analyses of Serum Biomarkers Related to OA

Serum was isolated, aliquoted and stored within 4 hours of blood collection at -80°C until biomarker analyses were performed. Serum biomarker analyses were repeated as necessary for samples with a $>15\%$ coefficient of variation (CV). We measured five OA-related serum biomarkers: two type II collagen biomarkers indicative of collagen synthesis (PIIANP, CPII); a type II collagen biomarker indicative of collagen degradation (C_2C); a glycoprotein biomarker (COMP) originating from cartilage, synovium and tendon, which is associated with spine and knee OA (Addison SR 2009) and impacted by synovitis (Vilim, Vytasek et al. 2001; Addison SR 2009); and a high molecular weight polysaccharide (HA), an excellent indicator of total body burden of OA including osteophyte and cartilage loss (Kraus VB 2009). When serum from more than one reunion was available, the most recent sample was used.

4.3.2.1 PIIANP (*type IIA collagen N-propeptide*)

PIIANP, a marker of a fetal form of collagen II recapitulated in OA, was measured by competitive ELISA (LINCO Research, St. Charles, MO, USA). The minimum detection limit is 17.2 ng/ml. Intra-assay and inter-assay CVs were $< 6.6\%$ and 7.8% , respectively.

4.3.2.2 CPII (type II procollagen carboxy-propeptide)

CPII, a marker of the adult form of collagen II synthesis, was measured by competitive ELISA (IBEX, Montreal, Quebec, Canada). The minimum detection limit is estimated to be 35.1 ng/ml. Intra-assay and inter-assay CVs were < 3.7% and \leq 9.1%, respectively.

4.3.2.3 C₂C (neoepitope from cleavage of Collagen II)

A competitive ELISA (IBEX) was used to measure the neoepitope produced by the cleavage of type II collagen (C₂C). The minimum detection limit is reported to be 7.3 ng/ml. Intra-assay and inter-assay CVs are < 2.4% and \leq 9.5%, respectively.

4.3.2.4 COMP (cartilage oligomeric matrix protein)

COMP was measured with an in-house sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (Clark, Jordan et al. 1999; Jordan, Luta et al. 2003), using monoclonal antibodies 17C10 (epitope in the epidermal growth factor-like domain) and 16F12 (epitope in the NH₂-terminal domain) against human COMP (Kong, Stabler et al. 2006). The minimum detection limit is 120 ng/ml. Intra-assay and inter-assay CVs were <5.8% and \leq 8.7%, respectively.

4.3.2.5 HA (hyaluronan)

HA was measured by enzyme-linked binding protein assay (Corgenix Inc. Westminster, Colorado, USA). The assay uses enzyme-conjugated hyaluronic acid binding protein (HABP) from bovine cartilage to specifically capture HA from human serum. The minimum detection limit is established at 10 ng/ml. Intra-assay and inter-assay CVs were <4.7% and $\leq 7.0\%$, respectively.

4.3.3 DNA isolation and quality control

DNA was isolated from buffy coat (derived from 5 ml fresh EDTA blood) (n=347) or 4 ml saliva (n=3). Saliva samples were obtained by mail when available blood was insufficient for DNA isolation but sufficient for serum biomarker analyses. DNA was extracted from blood and saliva using the PUREGENE DNA Purification Kit (Gentra Systems Inc, Minneapolis, Minn) per the manufacturer's instructions. DNA concentration was quantified by Thermo Scientific NanoDrop™ spectrophotometry (Wilmington, DE). DNA quality was verified on 0.8% agarose gels (0.8 g Seakem, 5 μ l Ethidium Bromide in 100ml 1x Tris-acetate-EDTA buffer), run at 90 V for one hour) using 0.5 μ l aliquots of each sample. A HindIII digest of lambda DNA (New England Biolabs, N3012S) was used as a reference ladder. DNA was scored 0 - 5 with score ≥ 4 indicating that a high molecular weight DNA band was clearly visible, and score <4 indicating that a high

molecular band was not clearly visible. Samples with a score ≥ 4 were used for whole-genome genetic mapping assays (n=349).

4.3.4 Whole-genome genotyping

The Infinium Human Linkage-12 Genotyping BeadChip (6,090 SNPs, Illumina, San Diego, CA) was used for whole-genome genotyping by fluorescence-based methods. The BeadChip included 6,090 SNP markers with an average spacing of 0.58 cM across the whole genome. As quality controls to insure accuracy for these assays, two blinded samplings from CEPH controls were genotyped for each plate. The genotype calls were analyzed by Illumina® Beadstudio Genotyping (GT) module software. A total of 98.8% (6,015 SNPs) of the 6,090 SNPs were successfully called. The distance from the telomere as estimated using the deCODE map.

4.3.5 Statistical analysis

Heritability (H^2_r), two-point and multi-point genome-wide quantitative trait locus (QTL) linkage scans for five OA-related biomarkers were estimated and performed using a variance components methodology as implemented in Sequential Oligogenic Linkage Analysis Routines (SOLAR, SFBR/NIH, San Antonio, TX) (Almasy and Blangero 1998). All models were adjusted for age and gender. Heritability of each OA-biomarker was estimated as the proportion of

phenotypic variation attributable to genetic variation using maximum likelihood methods. The significance of the variance components were tested using standard likelihood methods. To identify the candidate genomic regions that influence the OA-biomarker levels, the variance components model was expanded to partition the genetic variance into unobserved QTL, residual additive genetic, and residual non-genetic components according to the concepts of the variance components approach (Amos 1994).

Briefly, the variance component method models phenotypic covariances between arbitrary relatives as a function of the identity-by-descent (IBD) relationships at a given marker locus, assumed to be tightly linked to an additive locus influencing the quantitative trait (Almasy, Dyer et al. 1997). The IBD probabilities were computed using the Markov chain Monte Carlo algorithm as implemented in Loki for this extended complex pedigree (Heath 1997). The Loki IBD files were converted into SOLAR format for subsequent linkage analysis. The whole-genome linkage used IBD values calculated for 6,090 SNP markers. Biomarker data for all family members (including unaffected members) were included in the QTL analysis with the exception of seven participants: two with known rheumatoid arthritis to avoid confounding by other forms of arthritis; and five individuals younger than 25 years of age to avoid confounding by high cartilage biomarker concentrations due to cartilage growth plate turnover from skeletal immaturity. OA-biomarker concentrations were logarithmically transformed to achieve a normal distribution for SOLAR analyses. For those

biomarkers (CPII, HA) with residual kurtosis (RK) >1 , outliers were removed that exceeded 3 standard deviations from the mean (3 individuals from CPII and 6 individuals from HA). The resulting residual kurtosis (RK) for each biomarker was <0.7 . As recommended by SOLAR, the values for CPII, C2C and COMP were multiplied by a scaling factor (2.6, 3.8 and 2.4 respectively) to increase the standard deviation above 0.5. For all the biomarkers, linkage was considered significant if the LOD score exceeded 3.0. Results of $\text{LOD} \geq 1.5$, suggestive of linkage, are also reported (Hunter, Demissie et al. 2004).

4.4 Results

4.4.1 Heritability of biomarkers

Heritability estimates were performed with age- and sex-adjustment for each OA-related biomarker. After removing the outliers, biomarker and genetic marker data were available on 333 family members for COMP, PIIANP and C2C; 330 family members for CPII; and 327 family members for HA. The highest residual heritability (after the removal of age and gender effects) was observed for PIIANP (57%), followed by HA (49%), COMP (43%), and C2C (30%); CPII was not significantly heritable (3%). The four biomarker traits that were significantly heritable (p value <0.01) (Table 4.1), were used as quantitative traits in genome-wide linkage analyses using two-point and multiple-point models.

Table 4.1 Heritability (h^2) for Quantitative Traits of OA-related Biomarkers

OA endophenotypes	Numbers	Covariates	Mean \pm SD	h^2	p value	
COMP	333	Age, sex	17.73 \pm 1.07	0.43	0.001	COMP= cartilage oligomeric matrix protein, HA=hya luronan, PIIANP =type
HA	327	Age, sex	3.60 \pm 0.86	0.49	0.001	
PIIANP	333	Age, sex	7.17 \pm 0.52	0.57	<0.001	
CPII	330	Age, sex	18.36 \pm 0.97	0.032	0.4	
C2C	333	Age, sex	20.33 \pm 1.01	0.3	0.01	

IIA collagen N-propeptide, CPII=type II procollagen carboxy-propeptide, C2C=neoepitope from cleavage of CII

Heritability and p value were calculated from SOLAR

4.4.2 Genome-wide linkage analyses

4.4.2.1 Two-point linkage analysis

A total of 39 loci with LOD >1.5 were identified by two-point linkage analysis. The maximum LOD (3.1) was obtained using PIIANP, yielding linkage to chromosome 9q22.2 (rs2780701), which is within 2 Mb of the aspirin (ASPN) gene and within 3 Mb of the cathepsin L (CTSL) and Osteoglycin (OGN) genes. The next highest LOD score (2.7) was also obtained for PIIANP yielding linkage to chromosome 4q13.1 (rs1563796), which is close to the insulin-like growth factor binding protein 7 (IGFBP7) and ADAMTS3 genes. For COMP and HA, the maximum LOD scores were 2.23 on chromosome 14q24.2 (rs221924) and 1.79 on chromosome 1q25.3 (rs1020782), respectively. These are close to the type II deiodinase iodothyronine (DIO2) gene (less than 10 Mb away), and the prostaglandin-endoperoxide synthase 2 (PTGS2) gene (less than 5 Mb away),

respectively (Table 4.2). No LOD scores >1.5 were achieved by two-point linkage with C₂C.

Table 4.2 SNPs showing evidence for linkage (two-point LOD >1.5) in CARRIAGE Family

OA endo-phenotypes	Chromosome	Location (cM)	Genetic Marker	Peak LOD	Previously reported OA candidate genes near these regions*
PIIANP	1	133.27	rs1246194	1.7879	COL11A1
	2	167.91	rs964176	1.583	TNFAIP6, FAP
	4	34.5	rs1325107	1.7332	SOD3
		53.76	rs10023150	1.7051	SOD3
		76.25	rs1563796	2.6508	IGFBP7, ADAMTS3
	7	98.06	rs473880	1.5616	CD36
		179.01	rs6953751	1.8785	
	8	1.69	rs763869	1.7027	
		2.4	rs4242539	1.6619	
		7.79	rs3849827	1.8242	
	9	88.03	rs729958	2.5157	CTSL, ASPN, OGN
		94.51	rs2780701	3.0917	CTSL, ASPN, OGN
		95.55	rs1316268	1.72	CTSL, ASPN, OGN
		100.39	rs6478437	1.6689	CTSL, ASPN, OGN
		127.84	rs1013324	1.6983	EDG2
		128.7	rs4679	1.9338	
		128.74	rs1571586	2.2265	
		136.55	rs913275	1.8641	
		138.31	rs1220789	1.6528	
		154.29	rs2989726	1.8597	
	14	52.37	rs1950209	1.5489	ESR2
	15	132.59	rs2949	1.7772	AGC1
	16	38.48	rs1389504	1.5759	IL4R
	41.19	rs724307	1.6226	IL4R	
	57.05	rs1843609	1.7461	IL4R	
	57.1	rs11647994	1.5928	IL4R	
	57.85	rs17734120	1.6329		
17	10.04	rs149245	1.7593		
	15.04	rs7221818	1.7646		

		33.94	rs2240519	1.5641	
COMP	14	69.55	rs221924	2.2315	ESR2, DIO2
	16	14.53	rs1035564	1.5559	
	18	40.13	rs1893495	2.2207	
HA	1	181.52	rs1020782	1.79	PTGS2, PLA2G4A
	6	64.36	rs722269	1.7612	IL-17A, IL-17F, COL11A2, HLA
		101.6	rs1133503	1.6326	
		146.82	rs583341	1.5371	ESR1
	8	132.31	rs7814955	1.6093	TNFRSF11B
	19	63.42	rs4805201	1.5593	TGFB1

ADAMTS3=ADAM metalloproteinase with thrombospondin type 1 motif, 3;AGC1=aggrecan 1;ASPN=aspirin;CD36=platelet glycoprotein 4;COL11A1=collagen, type XI, alpha 1;COL11A2=collagen, type XI, alpha 2;CTSL=cathepsin L;DIO2= Type II iodothyronine deiodinase;EDG2= endothelial differentiation, lysophosphatidic acid (LPA) GPCR, 2;ESR1=estrogen receptor 1;ESR2= estrogen receptor 2;FAP=fibroblast activation protein, alpha;IGFBP7=insulin-like growth factor binding protein 7;IL4R=interleukin 4 receptor ;IL17A=interleukin 17A;IL17F=interleukin 17F;OGN=osteoglycin;PLA2G4A= phospholipase A2, group IVA;PTGS2= prostaglandin-endoperoxide synthase 2;SOD3=superoxide dismutase 3, extracellular;TGFB1=transforming growth factor, beta 1;TNFAIP6=tumor necrosis factor, alpha-induced protein 6;TNFRSF11B=tumor necrosis factor receptor superfamily, member 11b; LOD=Logarithm of odds

*** the previously reported OA candidate genes which are less than 10cM to the SNP (Meulenbelt, Min et al. 2008; Mototani, Iida et al. 2008; Ryder, Garrison et al. 2008; Valdes, Loughlin et al. 2008)**

4.4.2.2 Multi-point linkage analysis

Results of multipoint analysis of the genome-wide linkage scan are plotted separately for the four highly heritable OA-related biomarkers (Figure 4.1). A total of 23 loci (from 19 separate non-overlapping regions) with LOD >1.5 were identified (Table 4.3). Two significant linkage peaks (LOD >3) were obtained for chromosome 8, with PIIANP and COMP as quantitative traits (Figure 4.2A). The highest LOD score (4.33) was obtained using PIIANP, yielding linkage to chromosome 8p23.2 (near marker rs3849827). The next most significant LOD score (3.18) was obtained using COMP, yielding linkage to chromosome 8q11.1 (near marker rs7826304). Suggestive linkage overlapping these regions has been reported by Greig based on hand radiographic phenotypes (Greig, Spreckley et al. 2006). Another high LOD score (2.5) was obtained using COMP yielding linkage to chromosome 8q24.2 at 149cM (near marker rs2282); this linkage overlaps a region of linkage previously reported to Wnt-1-induced secreted protein 1 (WISP1) based on a spinal OA radiographic phenotype in postmenopausal Japanese women (Urano, Narusawa et al. 2007).

Chromosome 6 was notable for overlapping regions of linkage (LOD 1.69-2.25) in the interval 38-52 cM identified by PIIANP and HA (Figure 4.2B). This region corresponds to a report of linkage in a female UK cohort with hip OA (Southam, Dowling et al. 2004). Chromosome 14 was notable for overlapping regions of linkage (LOD 1.57-2.57) in the interval 59-82 cM identified by COMP and HA (Figure 4.2C). This region corresponds to a report of linkage in a UK cohort with hand OA (Greig, Spreckley et al. 2006). Overall, PIIANP yielded three

overlapping regions with HA, C₂C, and COMP on chromosomes 6, 13, and 15 respectively. For HA and C₂C, the highest LOD scores were obtained for additional regions on chromosomes 6q16 (LOD 1.96 for HA) and 5q31 (LOD 1.98 for C₂C). In addition to identifying previous reported OA candidate genes within or near our linkage peaks on chromosome 6, 8 and 14 (Figure 4.2 A-C), we also list potential candidates based on their potential biological relevance.

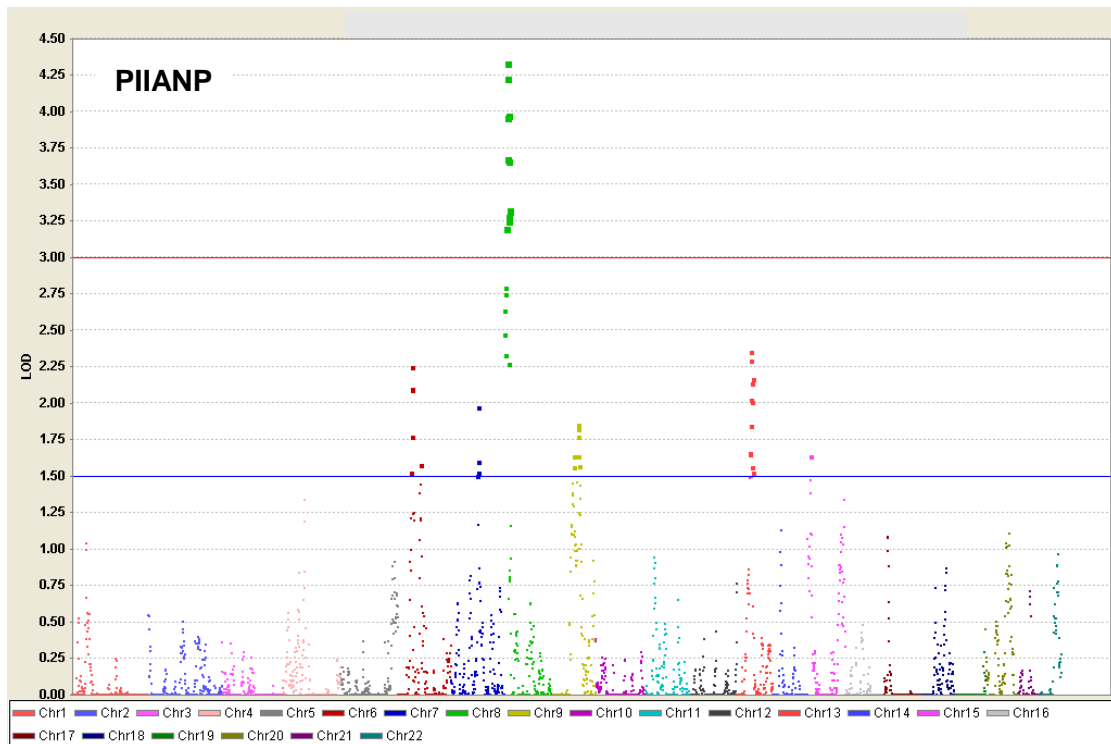


Figure 4.1A: The linkage signals (multipoint LOD scores) for the 4 highly heritable OA-related biomarkers in 22 chromosomes. A. PIIANP (type IIA collagen N-propeptide); significant linkage if $\text{LOD} \geq 3.0$ and suggestive of linkage if $\text{LOD} \geq 1.5$. All figure panels were generated with HaploView software

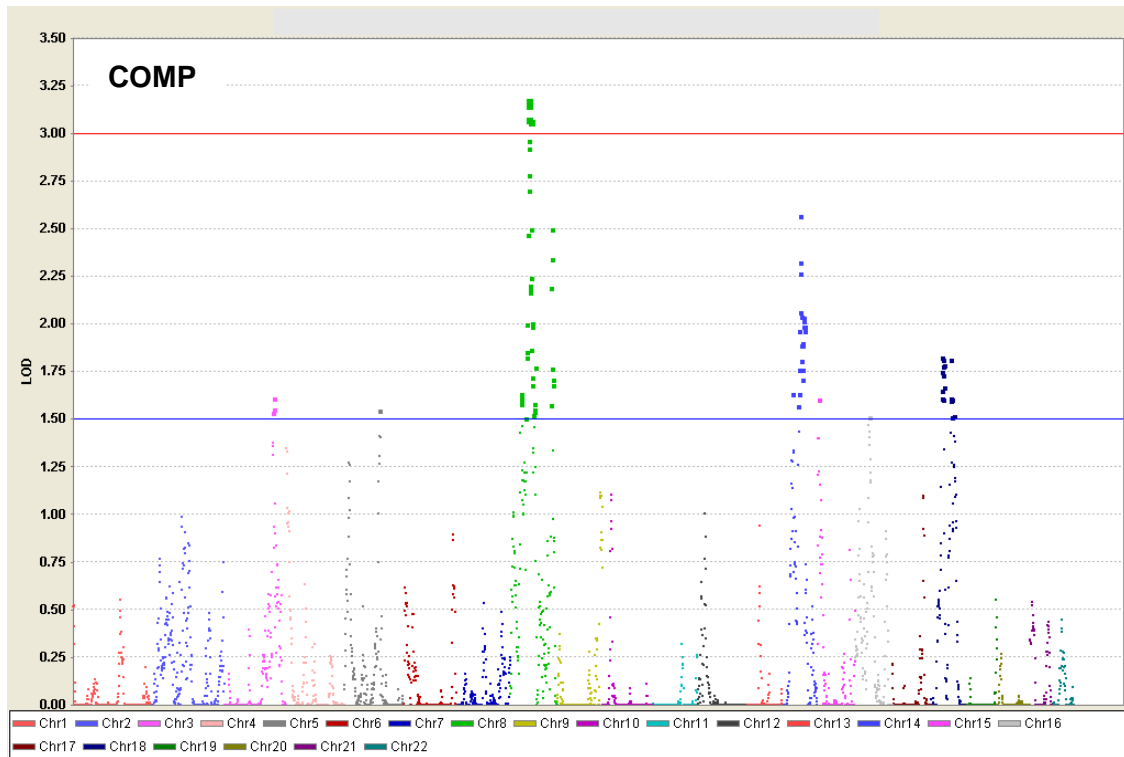


Figure 4.1B: The linkage signals (multipoint LOD scores) for the 4 highly heritable OA-related biomarkers in 22 chromosomes. B. COMP (cartilage oligomeric matrix protein).

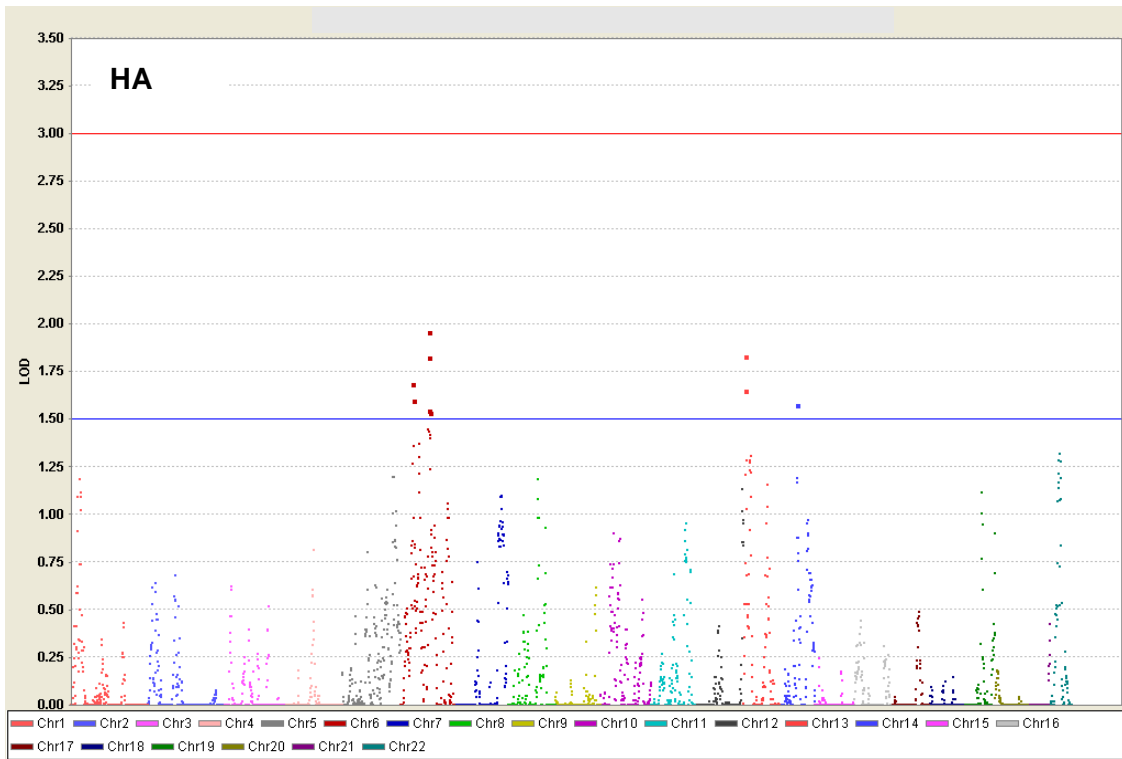


Figure 4.1C: The linkage signals (multipoint LOD scores) for the 4 highly heritable OA-related biomarkers in 22 chromosomes. C. HA (hyaluronan)

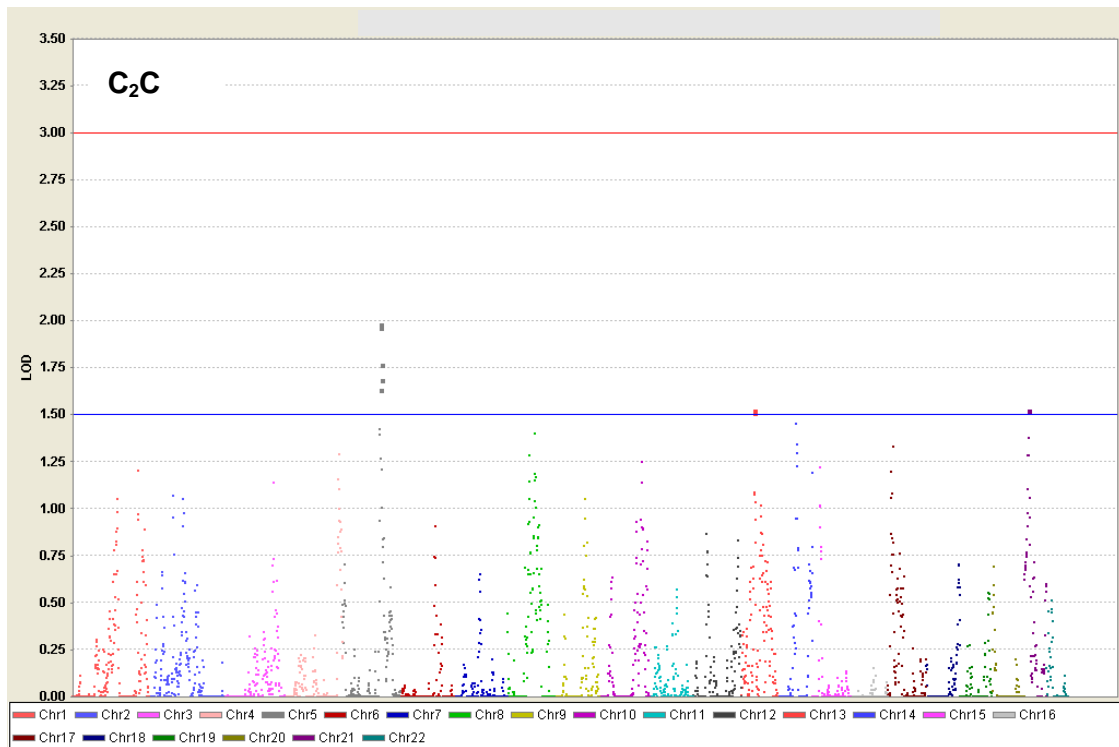


Figure 4.1D: The linkage signals (multipoint LOD scores) for the 4 highly heritable OA-related biomarkers in 22 chromosomes. D. C₂C (neoepitope from cleavage of CII)

Figure 4.2A

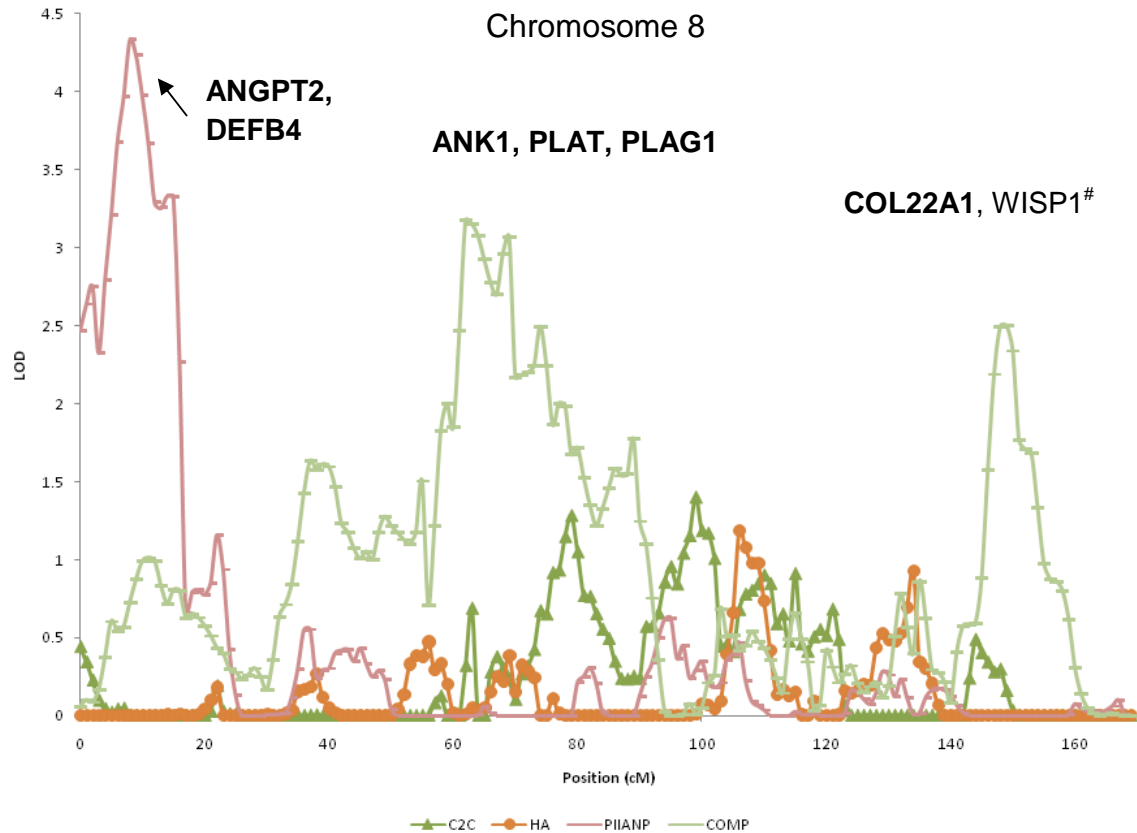


Figure 4.2A-C: Potential OA candidate genes in chromosome 6, 8, and 14.

Genes listed in bold over the peaks represent potential novel candidate genes associated with biomarkers of OA in this study that have potential biological relevance for OA based on the literature. Genes listed without bold type over the peaks represent candidate genes linked previously to OA. The # sign indicates that the genes are less than 10 cM from the border of the 1-LOD drop support interval. ANGPT2=angiopoietin 2; DEFB4=beta-defensin 4; ANK1=ankyrin 1;

PLAT=plasminogen activator, tissue; PLAG1= pleomorphic adenoma gene 1;
 COL22A1=collagen, type XXII, alpha 1; WISP1=wnt-1-induced secreted protein 1;
 HFE=hemochromatosis; COL11A2=collagen, type XI, alpha 2; TNF=tumor
 necrosis factor; COL10A1=collagen, type X, alpha 1; ESR2=Estrogen receptor 2;
 DIO2=Type II iodothyronine deiodinase; FLRT2=Fibronectin leucine-rich
 transmembrane protein 2.

Figure 4.2B

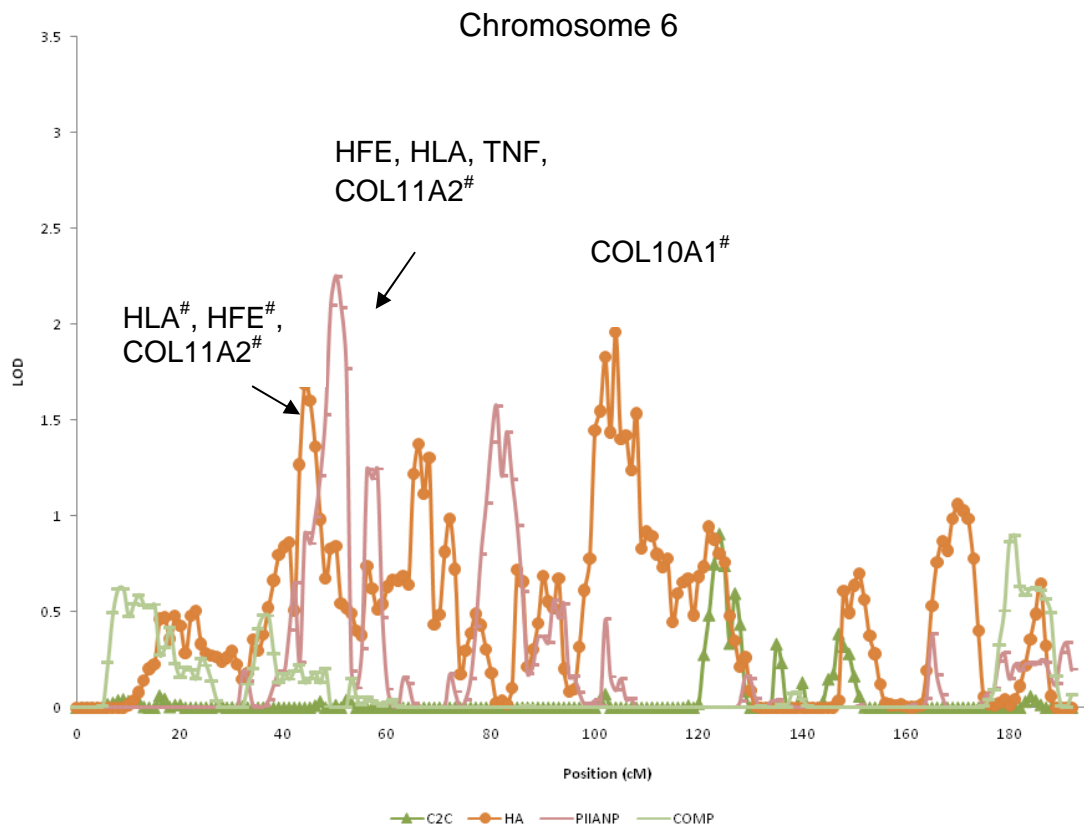


Figure 4.2C

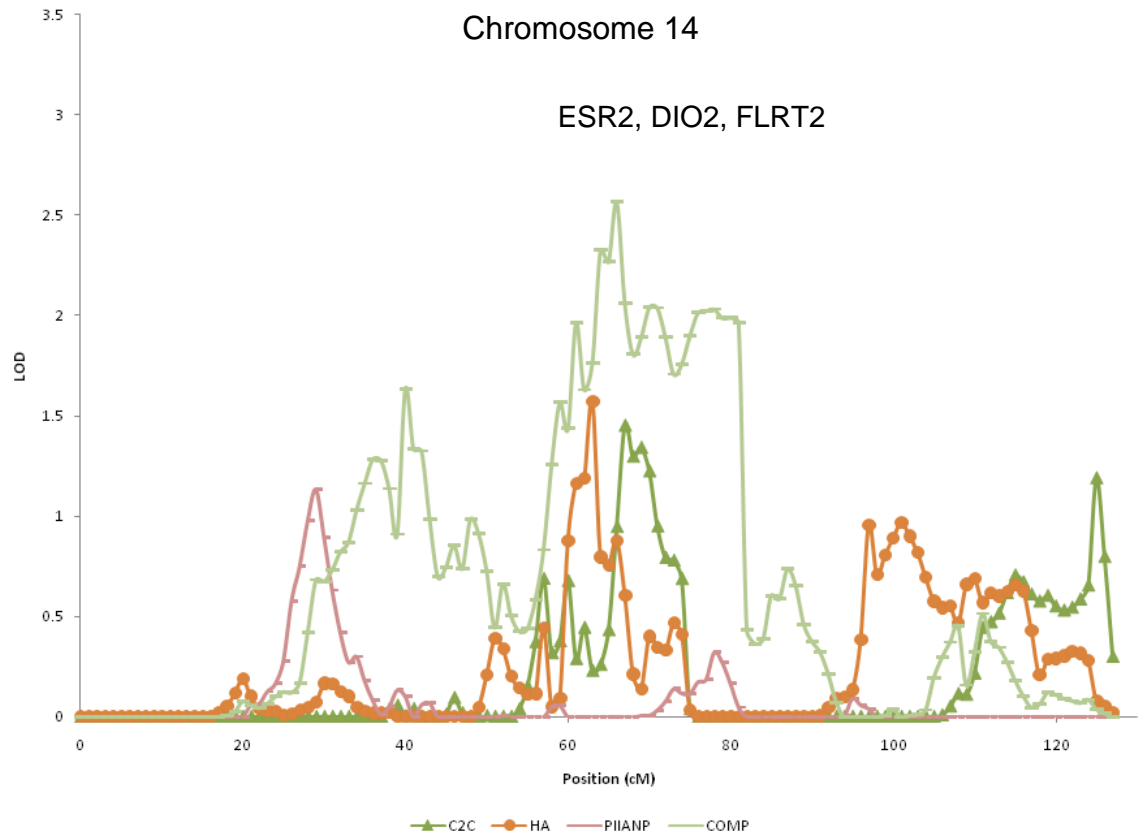


Table 4.3 Regions of linkage (multipoint LOD >1.5) to quantitative traits in CARRIAGE Family with citation of studies reporting OA associations that overlap these regions

OA endo-phenotype	Chromosome	Multipoint LOD	Location of peak LOD score (cM)	1-LOD interval (cM)	Overlapping biomarkers	Previously reported OA candidate genes in these region*	Previous reported OA linkages overlapping these regions				
							Phenotype	Peak LOD	Distance (cM)	Population	Ref
PIIANP	6	2.2504	50	47-52	HA	HFE, HLA, TNF, COL11A2 [#]	Female Hip	4.8	53-56	UK	(Southam, Dowling et al. 2004)
	7	1.97	98	96-102		CD36 [#]					
	8	4.3341	8	5-15			Hand JSN	1.57	8.3-21.3	UK	(Greig, Spreckley et al. 2006)
	9	1.6385	100	72-108		CTSL, ASPN, OGN, EDG2 [#]	Hand JSN-sum	2.3	76	USA	(Demissie, Cupples et al. 2002)
COMP	13	2.3492	50	45-57	C2C	LRCH1					
	15	1.6337	9	0-14	COMP						
	3	1.6079	179	173-183							
	5	1.548	127	121-129							
	8	3.1764	62	60-70			Hand U-DIP	2.56	41.5-79.4	UK	(Greig, Spreckley et al. 2006)
	8	2.4991	149	145-155		WISP1					
	14	1.6339	40	29-52			Hand U-DIP	1.44	40.5-47.5	UK	(Greig, Spreckley et al. 2006)
	14	2.5663	66	59-82	HA	ESR2, DIO2, FLRT2, CALM1 [#]	Hand U-JSN	2.64	48-57	UK	(Greig, Spreckley et al. 2006)
	15	1.6039	6	2-20	PIIANP						
	16	1.5078	50	45-68		IL4R	Early-onset	2.6	28-47	Iceland	(Ingvarsson,

						Hip				Stefansson et al. 2001)	
						Female Hip	1.7	46	UK	(Forster, Chapman et al. 2004)	
						Hand U-JSN	2.64	48.5-57.8	UK	(Greig, Spreckley et al. 2006)	
	18	1.8108	45	36-49							
	18	1.8103	72	65-91		Hand OST	1.34	71-85	UK	(Greig, Spreckley et al. 2006)	
						Knee OA	2.41	60.1-86.1	US/UK	(Jordan 2008)	
C2C	5	1.9784	139	133-144		SLC26A2 [#]					
	13	1.5112	45	41-51	PIIANP	LRCH1, KLOTHO [#]	Hand OST	1.28	17.2-25.1	UK	(Greig, Spreckley et al. 2006)
							Hand K/L sum	1.6	36	USA	(Demissie, Cupples et al. 2002)
	21	1.5235	15	6-22							
HA	6	1.6873	44	38-48	PIIANP	HLA [#] , HFE [#] , COL11A2 [#]	Female Hip	4.8	53-56	UK	(Southam, Dowling et al. 2004)
	6	1.9559	104	100-108		COL10A1 [#]	Hand U-OST	1.11	82.6-109.9	Netherlands	(Stefansson, Jonsson et al. 2003)
	13	1.829	7	5-11		KLOTHO [#]					
	14	1.5741	63	60-67	COMP	ESR2	Hand U-JSN	2.64	48-57	UK	(Greig, Spreckley et al. 2006)

ASPN=asporin;CALM1=calmodulin 1;CD36=platelet glycoprotein 4;COL10A1=collagen, type X, alpha 1;COL11A2=collagen, type XI, alpha 2;CTSL=cathepsin L;DIO2= type II iodothyronine deiodinase;EDG2= endothelial differentiation, lysophosphatidic acid (LPA) GPCR, 2;ESR2=estrogen receptor 2;FLRT2= fibronectin leucine rich transmembrane protein 2;HFE=hemochromatosis;IL4R=interleukin 4 receptor;LRCH1=leucine-rich repeats and calponin homology domain-containing 1;OGN=osteoglycin;SLC26A2=solute carrier family 26;TNF=tumor necrosis factor;WISP1=wnt-1-induced secreted protein 1
JSN=joint-space narrowing;OST=osteophyte;JSN-sum=sum of joint space narrowing scores;K/L sum=sum of Kellgren/Lawrence scores;U-DIP=unaffected distal interphalangeal;U-JSN=unaffected joint-space narrowing;
U-OST=unaffected osteophyte; LOD=Logarithm of odds

indication of genes are less than 10cM from the border of the 1-LOD drop support interval

* the previously reported OA candidate genes are described in references

(Urano, Narusawa et al. 2007; Meulenbelt, Min et al. 2008; Mototani, Iida et al. 2008; Ryder, Garrison et al. 2008)

4.5 Discussion

This is the first linkage study to identify genetic loci associated with OA using biological markers. In the previous five genome-wide linkage studies from multiple relative or affected sibling-pair families, OA phenotypes were uniformly defined by X-ray evidence, physical examination, or joint replacement, which detect late stages of OA (Chapman, Mustafa et al. 1999; Leppavuori, Kujala et al. 1999; Loughlin, Mustafa et al. 1999; Demissie, Cupples et al. 2002; Stefansson, Jonsson et al. 2003; Hunter, Demissie et al. 2004; Greig, Spreckley et al. 2006). There have been few studies investigating the heritability of OA biomarkers. In previous UK twin and GARP siblings-pair studies, the heritability of COMP and PIIANP were significant, 40-70% and 62% respectively (Williams, Andrew et al. 2006; Meulenbelt, Kloppenburg et al. 2007). The genetic components were in agreement with our findings, despite different race and study designs (Williams, Andrew et al. 2006; Meulenbelt, Kloppenburg et al. 2007).

The validity of our overall strategy was borne out by our replication of several previously reported genetic associations with OA identified by other means of phenotyping (Table 4.3). Overall, we identified 14 regions of linkage to OA-related quantitative traits that overlap or are near (within 10 cMs) regions with reported genetic association with OA in the current literature. In addition, this study provides evidence for two novel OA loci on chromosome 8 based on PIIANP and COMP quantitative traits. A previous study using radiographic phenotypes reported suggestive linkage (LOD 1.57 and LOD 2.56) (Greig,

Spreckley et al. 2006) at loci overlapping the two regions found here, but candidate genes have not yet to be identified for these regions. Several of the OA-associated loci identified in this study were detected by more than one biomarker trait. This supports our hypothesis that a panel of biomarkers could identify shared genetic determinants. Loci identified by more than one OA-related biomarker may be of particular interest for further study as they are less likely to represent false positives and more likely to represent genes regulating the whole joint organ.

Of note, we did not detect linkage to several genes with known OA association including frizzled-related protein β (*FRZB*), growth differentiation factor 5 (*GDF5*), and von Willebrand factor A domains (*DVWA*). All three of these proteins are related to skeletal morphogenesis and bone morphogenic cell signaling (Loughlin, Dowling et al. 2004; Miyamoto, Mabuchi et al. 2007; Miyamoto, Shi et al. 2008). Our biomarker panel did not include a primary bone marker and so may have failed to account for the metabolic pathways impacted by these genes. These seminal studies have been performed in Caucasian or Asian populations while our study was performed in individuals of mixed African American and Native American heritage; thus, ethnic variation in genetic etiologies of OA may in part account for the failure to detect these loci in our cohort. Statistical power may also be an issue, as these three studies included between 1696 and 4361 individuals. Finally, our study was conducted in one

large extended family, and it would not be reasonable to expect that every possible genetic etiology of OA would be reflected in this one family.

A strength of this study is that it is based on data from a large extended family with a pedigree spanning 300 years and 10 generations from a single founder. Statistical power can be increased by the use of biomarkers as quantitative traits (Wallace, Newhouse et al. 2008). Increased statistical power may also come from minimizing genetic variability through study of a cohort from a single founder. A limitation of the study, however, was the inability to perform radiographic phenotyping due to the health fair setting in which individuals were ascertained. Nevertheless, our biomarker traits led to replication of several loci reported in previous OA genetic studies that used radiographic phenotyping. Also, we have previously shown that several of the OA biomarkers used here were associated with clinical OA phenotypes in this large multigenerational family (Chen, Shah et al. 2008). In addition, all the biological markers (PIIANP, COMP, C₂C, HA and CPII) have been associated with OA in other studies (Bauer, Hunter et al. 2006) .

In summary, we report the first evidence for OA linkage using quantitative biomarker traits in a large extended family. We not only replicated several loci reported in previous OA genetic studies, but also identified two significant novel loci on chromosome 8. Several of the loci were identified by more than one OA-related biomarker. Further studies of the candidate genes at these loci may

provide new insights into the mechanisms of joint metabolism, and OA initiation and progression.

4.6 Heritability and Linkage Results of Joint Hypermobility

4.6.1 Introduction

Cartilage oligomeric matrix protein (COMP), a homopentameric extracellular matrix glycoprotein, is synthesized in cartilage as well as in tendon and ligament.

Joint hypermobility due to ligamentous laxity is one of the clinical heritable traits in some osteochondrodystrophies, which are associated with profoundly low serum COMP due to COMP mutations. In our previous study (chapter 3), we found that general joint hypermobility was associated with decreased Osteoarthritis (OA) risk and lower serum COMP levels; however, the precise mechanism of this association remains unclear. The aim of this investigation was to estimate the heritability of serum levels of COMP and joint hypermobility and identify genetic susceptibility loci for joint hypermobility in this large extended family.

4.6.2 Methods

(Please refer to 4.3.1 for the study population, 4.3.4 for whole-genome genotyping, and 4.3.5 for QTL mapping; please refer to 3.3.5 for hypermobility definition)

4.6.3 Results and conclusions

The estimates of the heritability for serum COMP and Beighton scores are depicted in Table 4.4. We found evidence of substantial heritability for serum COMP levels as well as joint hypermobility based on Beighton scores. Our finding agrees with the previous study showing an estimated 40% heritability for COMP in a UK female twin cohort (Williams, Andrew et al. 2006). Our study also shows joint hypermobility is highly heritable in this extended family. The level of heritability of these two traits (between 35-44%) indicates that both joint hypermobility and serum COMP are reasonable phenotypes for further quantitative trait linkage analysis. Summary of the linkage SNPs and peaks by two-point and multi-point analysis for quantitative joint hypermobility traits are shown in Table 4.5 and 4.6.

Table 4.4 Heritability of Joint hypermobility and serum COMP

Phenotype	COMP	Beighton Scores
Residual Heritability (%)	43.2	35.1
(p-value)	(0.001)*	(<0.05)*
Covariate		
Age (p-value)	(1.6291357e-10)*	0.3950335
Sex (p-value)	(0.0091972)*	(0.0058804)*

Table 4.5 Summary of the linkage SNPs (two-point LOD > 1.5) for joint hypermobility in the CARRIAGE family

Chromosome	SNP	LOD
1	RS204057	1.5323
	RS1436171	1.5699
	RS1795030	1.7334
3	RS749932	1.522
	RS795734	2.2248
5	RS34638	2.3204
	RS547356	1.5985
	RS1200485	1.9123
	RS1363614	2.0581
	RS1460039	1.6022
	RS2545387	1.643
10	RS1034178	1.6439
11	RS485345	1.5367
12	RS965125	1.5343
	RS2043623	1.5419
	RS722952	1.5103
13	RS1176270	1.5656
	RS1332470	2.0952
	RS276855	1.8007
15	RS1420040	1.5874
16	RS7195006	1.814
	RS680798	1.5484
18		

Table 4.6. Summary of the linkage peaks (multi-point) for joint hypermobility traits in the CARRIAGE family

Chromosome	Position (cM)	Peak LOD score
1	39	1.3148
2	94	1.8477
	89	1.5181
	95	1.8261
	96	1.6859
3	25	1.5198
4	158	0.5775
5	24	1.0386
6	89	1.1631
7	80	1.0032
8	0	0.8677
9	142	1.0097
10	132	1.5817
	133	1.578
11	65	0.6099
12	13	0.9757
13	73	0.4375
14	105	0.5463
15	31	0.1223
16	68	0.7679
17	21	0.7558
18	9	0.4297
19	113	0.3248
20	67	1.2194
21	46	0.5151
22	21	1.3118

CHAPTER 5

Conclusion and Future directions

5.1 Thesis Conclusions

Our major hypothesis (See Chapter 2 and 4) for this project was that OA biomarkers could be used as quantitative traits in this extended family, and would provide enough power to identify novel or replicate known genetic loci for OA. In order to test this hypothesis, we have finished five sub-aims. First, we established this multigenerational pedigree including 3327 family members. Second, we defined OA by three clinical criteria (ACR, GOGO and any single hand joint involvement), and hand symptoms. Third, we determined and evaluated seven potential OA-related biomarkers in this big family. Fourth, we isolated DAN and performed whole-genome genotyping for those available family members. Lastly, we estimated heritabilities of biomarkers and identified genetic susceptibility loci for OA-related biomarkers.

Our second hypothesis (See Chapter 3 and 4) was based on the inverse relationship between joint hypermobility and OA in GOGO study (Kraus, Li et al. 2004). We hypothesized that joint hypermobility reduced OA risk and could serve as a quantitative trait for identifying protective loci for OA. In order to test this hypothesis, we have finished four sub-aims. First, we used the family information

from our first hypothesis. Second, we defined joint hypermobility according to the Beighton criteria and surveyed joint symptoms in this big family. Third, we used the whole-genome genotyping data from our first hypothesis. Lastly, we estimated heritability of joint hypermobility and identified genetic susceptibility loci for joint hypermobility.

During our study, we not only found the inverse association between joint hypermobility and hand, knee OA in the CARRIAGE family, but also coincidentally discovered that joint hypermobility family members had significantly lower serum COMP levels compared with non-hypermobility individuals in this big family. This result was further validated in another GOGO population, which provided extensive radiographic OA phenotypes (Chen, Shah et al. 2008).

The summary of conclusions of this thesis can be listed as follows:

- 1) Serum biomarkers (HA, COMP, GSP, hs-CRP, PIIANP & HA/PIIANP ratio) can reflect clinical hand OA.
- 2) Hypermobility is associated with decreased prevalence of clinical and radiographic hand OA and clinical knee OA.
- 3) Hypermobility is associated with lower serum COMP in OA and non-OA cohorts

- 4) Four of five biomarkers showed significant heritable components (PIIANP 57%, HA 49%, COMP 43%, and C₂C 30%).
- 5) Joint hypermobility also has been shown to be highly heritable (35.1%, $p < 0.05$) in this extended family.
- 6) For OA-related biomarkers, thirteen of the 19 loci with multi-point LOD scores >1.5 were near or overlapped previously reported OA susceptibility loci.
- 7) Two significantly novel OA loci were found on chromosome 8 based on PIIANP and COMP quantitative traits (LOD 4.33 and 3.18, respectively). These two loci have been previously reported to have suggestive linkage (LOD 1.57 and LOD 2.56) using radiographic phenotypes.
- 8) The highest LOD score for joint hypermobility using Beighton scores as quantitative traits is on chromosome 2 (LOD 1.85 at 94 cM).

5.2 Future Directions

5.2.1 OA Biomarkers

5.2.1.1 Identifying disease-specific biomarkers

Although the concept of balance between synthesis and degradation of the ECMs for OA is well-accepted, no single biomarker has yet shown strong enough evidence to predict or monitor OA patients in clinics. When OA occurs, the

complicated interactive process among the entire joint (such as articular cartilage, synovium, ligaments, and subchondral bone) still needs to be elucidated comprehensively (Goldring and Goldring 2007). Moreover, little is known about the interactions between those cleaved matrix molecules or fragments and other molecules which involve the OA process simultaneously such as cytokines, chemokines, and so forth. We also know little about the source of biochemistry markers. For instance, serum hyaluronan (sHA) has its ubiquitous characteristic in connective tissues throughout the body (Criscione, Elliott et al. 2005). Likewise, serum COMP may come from cartilage, tendon, and synovium (Muller, Michel et al. 1998). Therefore, we might need to understand any tissue specificity of these biomarkers. In addition, biomarker concentrations in body fluid compartments are influenced by many critical processes such as the metabolism rate of the liver and the clearance of rate of the kidney (Simkin and Bassett 1995). This is because the increased level of a certain biomarker may come from increased synthesis, increased cleavage, and even from decreased clearance of renal functioning. These all make OA biomarkers difficult to apply to clinical diagnosis.

Despite many studies which have announced that some biomarkers establish better sensitivity to detect early OA than traditional radiologic techniques (Dragomir, Kraus et al. 2002), we are still so limited in our understanding of the interactions between these OA biomarkers and food and drug intake (Rossler, Laszlo et al. 1998; Gineyts, Mo et al. 2004; Kraus 2006).

Many studies have also shown that some biomarkers had diurnal variation (Criscione, Elliott et al. 2005; Kong, Stabler et al. 2006). Therefore, to understand and characterize OA development and progression, there is a need to discover accurate biological markers for OA diagnostic and prognostic measures in clinical medicine. Although many biomarkers in the CARRIAGE family study have been shown to be associated with clinical phenotypes, the specificity is still not satisfying due to huge overlapping regions between affected OA and normal individuals. Recent techniques such as high-resolution nuclear magnetic resonance (NMR) have shown 100% sensitivity and specificity to detect epithelial ovarian cancer (Odunsi, Wollman et al. 2005). This technology might provide a promising methodology for OA biomarkers.

5.2.1.2 Validation biomarkers from different races

Validating biomarkers as surrogate end points is a challenging process. It usually requires a long time to test the same assay showing the same result repeatedly in different populations, and races. Especially with OA, a complex disease with multiple joint sites of hands, knees, hips and spine, the task becomes almost formidable. Most of the biomarkers that we studied in the CARRIAGE family had been reported as potential biomarkers in Caucasian populations. In the CARRIAGE family, a mixed Native and African American family, we also showed that some biomarkers are associated with OA

phenotypes. However, there are limited reports of further validation for those biomarkers in an Asian population. Therefore, PIIANP and HA would be good candidate biomarkers to further validate in the Taiwanese population. In addition, GSP should be further evaluated in other populations to confirm the association with OA symptoms.

5.2.1.3 Biomarkers: detecting OA biological process or an OA risk factor?

PIIANP is highly heritable (57%) in our study, which also has been shown to be favorably inherited (62%) in the GARP study (Meulenbelt, Kloppenburg et al. 2007). Collagen IIA was mainly from chondroprogenitor cells of developing skeletal tissues. The mature articular chondrocytes do not secrete type IIA procollagen, but secrete splicing alternative form type IIB procollagen (Sandell, Morris et al. 1991), although studies also demonstrated that some adult chondrocytes might return to a chondroprogenitor phenotype in the process of a repair attempt (Aigner, Zhu et al. 1999; Salminen, Vuorio et al. 2001; Zhu, McAlinden et al. 2001). Interestingly, using principal component analyses for clusters of biomarkers, Meulenbelt et al suggested that circulating PIIANP may mainly come from hand and spine OA, rather than from knee or hip damage (Meulenbelt, Kloppenburg et al. 2007). OA definitely is not a single disorder, but overlapping distinct diseases. Therefore, identifying a high risk group among a normal population is an alternative optimal goal for biomarkers. Thus, whether

the serum PIIANP level represents an OA biological process or actually is an underlying risk factor of OA may need to be further addressed.

5.2.2 Genetics of OA

5.2.2.1 Fine mapping of the two significant loci on chromosome 8

We found two significant novel loci on chromosome 8, which also has been shown to have suggestive linkage peaks by previous OA radiographic phenotypes (Greig, Spreckley et al. 2006). Therefore, fine mapping with more dense SNP markers covering these 2 loci should be our next reasonable approach. Especially, under these two peaks, some potential candidate genes have been demonstrated to have OA relevance from biological studies based on the literature.

5.2.2.2 Candidate regions with pleiotropic effects

Another promising candidate locus is on chromosome 14. Likewise, Greig et al. showed an overlapping region similar to ours from the UK cohort study (Greig, Spreckley et al. 2006). In our study, biomarkers of COMP, HA, C₂C, hs-CRP all reveal some overlapping regions between 59 to 82 cM. This pleiotropic effect may imply that one gene or some genes under these overlapping peaks may influence the four biomarkers simultaneously. Recently, Meulenbelt et al also

reported a common coding variant that changed the amino acid (rs225014) of the deiodinase, iodothyronine, type II (DIO2) increasing the symptomatic OA risk from three cohorts (in the UK, Holland and Japan) (Meulenbelt, Min et al. 2008). The product of the DIO2, the D2 enzyme, converts intracellular T4 into the active T3, thereby inhibiting chondrocyte proliferation and stimulating chondrocyte differentiation (Bassett and Williams 2003). As a matter of fact, biomarkers of COMP, HA and hs-CRP are related to joint inflammation (Vilim, Vytasek et al. 2001; Sturmer, Brenner et al. 2004; Elliott, Kraus et al. 2005). Thus, further evaluation of the association between coding SNPs of the DIO2 gene and these biomarkers could possibly provide some understanding about symptomatic OA.

Furthermore, we identified some overlapping evidence for linkage (LOD >1.5) from at least 2 biomarkers on chromosome 5, 6, 13, and 15. In theory, indeed, biomarkers can reflect quantitative and dynamic changes of joint remodeling. Therefore, finding candidate loci or genes using a combination of different biomarkers might promise other exciting future directions and identify dynamic processes within the entire joint for OA. Lastly, there were no bone biomarkers (type I collagen such as CTX-1, NTX-1, pyridinoline, and osteocalcin) that we determined in our study. FRZB, GDF5 and DVWA are promising OA candidate genes, but do not obtain high linkage signals in our study. As we know, functions of these three proteins are related to skeletal morphogenesis and bone morphogenic cell signaling (Loughlin, Dowling et al. 2004; Miyamoto, Mabuchi et

al. 2007; Miyamoto, Shi et al. 2008); thus, genetic mapping for bone biomarkers might offer some interesting points in the CARRIAGE family.

5.3 Genetics and Biomarkers of Joint Hypermobility

We identified joint hypermobile family members with lower serum COMP compared with non-hypermobile individuals in the CARRIAGE family. However, using hypermobility quantitative traits (0-9) computed from SOLAR[®] with t-dist and lodadj (LOD adjustment) functions, we didn't find significant linkage signal covering the COMP gene in chromosome 19. The functions of t-dist and lodadj provided from SOLAR[®] are to resolve non-normal distribution data with high residual kurtosis. A recent study of an Icelandic group identified linkage of joint hypermobility to chromosome 19P 13.3 (LOD 3.8; within 16 Mb of the COMP gene) using binary traits rather than quantitative trait mapping. Therefore, binary trait mapping for joint hypermobility would be an alternative choice when the Beighton scores (0-9) cannot be distribution normalized.

We also performed direct sequence for those 26 mostly hypermobile family members from this extensive family, although this task has not yet been finished yet (exon 7, 9, 13 not finished). We have identified six novel SNPs in the COMP gene. We also found a coding SNP (RS61752496; Q756R) mutation in the six hypermobile individuals among these 26 people, but this SNP's function remains

unclear in the genome browsers. Therefore, understanding the associations between these SNPs with joint hypermobility and COMP levels would be helpful in future exploration of the mechanisms of joint hypermobility.

Lastly, so far, there are not any reports about COMP levels and joint hypermobility in the literature except ours. Based on our studies and some previous European small case-control studies, we hypothesize that joint hypermobility with low serum COMP would be a world-wide phenomene rather than restricted to Caucasians, Africans and Native-Americans. Actually, hypermobility is more frequent in Asians than in black and white populations (Grahame 1999). Last December, I also submitted a Taiwanese NSC (National Science Council) proposal to validate the COMP level in the Taiwanese Han population. Hopefully, finding the susceptibility alleles for some benign subgroups of joint hypermobility may help us in identifying alleles that are protective against OA.

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BIOGRAPHY

Education:

1990/8-1997/7 M.D. National Defense Medical Center, Taipei, Taiwan, R.O.C

Position and Employment:

1997/8-1999/7 Medical physician at Navy

1999/8-2002/7 Resident physician, Dept. of Internal Medicine, Tri-Service
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2002/8-2003/7 Senior resident and clinical fellow in division of Rheumatology,
Immunology and Allergy, Department of Medicine,
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2003/7-2004/7 Chief resident in department of Medicine,
Chief resident in division of Rheumatology, Immunology and
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2004/8-2005/7 Attending physician in division of Rheumatology, Immunology
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Board Certificate:

1997 Diplomate, Taiwanese Board of Medical Examiners (No.28329)

2002 Diplomate, Taiwanese Board of Internal Medicine (No.6176)

2004 Diplomate, Taiwanese Board of Allergy and Immunology (No.339)

2005 Diplomate, Taiwanese Board of Rheumatology (No.256)

Professional Societies:

Taiwan Society of Internal Medicine

Taiwanese Society of Immunology

Taiwanese Society of Rheumatology

Osteoarthritis Research Society International (OARSI)

The American Society of Human Genetics (ASHG): Training member

The American College of Rheumatology: International Fellow member

Honors and Awards:

1. Student chairperson, National Defense Medical Center, 1995
2. One of Best Interns, Tri-Service General Hospital, 1997
3. Medical Officer for Taiwanese Contractor Ship Transfer Trng, DS Charleston SC, USA, 1998/08-1998/12
4. Best Poster, awarded by the Taiwanese Society of Immunology 2002
5. Annual Excellent Clinical Research Publications, awarded by Tri-Service General Hospital 2004
6. Scholarship 2005/8-2009/7, provided by Taiwan's government

Ad Hoc Journal Reviewer

Arthritis and Rheumatism: since 2008

Workshops, and professional meetings:

1. Genetic Analysis of Complex Human Diseases, June 11-15, 2006, Center for Human Genetics, Duke University Medical Center
2. Models and Technologies for Defining Phenotype, July 31, 2006-August 11, 2006, National Institutes of Health Roadmaps Course, Wake Forest University Baptist Medical Center
3. Duke Bioinformatics Workshop, August 24-25, 2006, Duke Center for Human Genetics
4. Duke Molecular Biology Techniques Workshop, May 16-25, 2007, Duke University Medical Center, Durham, NC
5. Duke Bioinformatics Workshop, August 20-22, 2007, Duke Center for Human Genetics
6. A Practical Guide to Genome-Wide Association Workshop, March 18-19,

- 2008, IGSP, Duke University
7. Gordon Research Conference: Musculoskeletal Biology & Bioengineering, July 27-Aug 1, 2008, Proctor Academy, Andover, NH
 8. Duke Bioinformatics Workshop, August 18-20, 2008, Duke Center for Human Genetics
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PUBLICATION

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2. **HC Chen**, JH Lai, SY Kuo, YG Tsai, SH Lin, DM, Chang. An Unusual Cause of Obstructive Uropathy due to Lupus Interstitial Cystitis. Journal of Medical Sciences 2003; 23:127-130.
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4. YG Tsai, JH Lai, SY Kuo, **H-C Chen**, HL Wan, and DM Chang. Ruptured renal microaneurysms Complicated with a retroperitoneal abscess for a patient with systemic lupus erythematosus. Lupus 2003; 12:317-320. (SCI)
5. DM Chang, SJ Chu, **HC Chen**, SY Kuo, JH Lai. Dehydroepiandrosterone suppresses interleukin 10 synthesis in women with systemic lupus erythematosus. Ann Rheum Dis. 2004; 63:1623-6. (SCI)
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7. **HC Chen**, CM Shih, JH Lai, LL Chao, SY Kuo, DM Chang. Pleural effusion as a manifestation of Lyme disease. Journal of Rheumatology 2004; 31:811-3. (SCI)
8. **HC Chen**, WY Kao, DM Chang, HW Gao, WY Lai, JH Lai. Neutrophilic panniculitis with myelodysplastic syndromes presenting as pustulosis. American Journal of Hematology 2004; 76:61-5. (SCI)
9. **HC Chen**, CS Tsai, JT Lee, CA Chen, FY Chang. Acute quadriplegia complicating critical illness polyneuropathy in a patient with infective endocarditis: a case report. Journal of Infection 2005; 50:153-7. (SCI)
10. **HC Chen**, JH Lai, CH Chen, SY Kuo, DM Chang. Systemic Lupus Erythematosus with simultaneous onset of Kikuchi-Fujimoto's disease

- complicated with antiphospholipid antibody syndrome. Rheumatology Int. 2005; 25:303-6. (SCI)
11. TY Hou, DM Chang, HW Gao, CH Chen, **HC Chen**, JH Lai. Sweet's syndrome as an initial presentation in systemic lupus erythematosus: a case report and review of the literature. Lupus 2005; 14:399-402. (SCI)
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 13. FC Liu, **HC Chen**, DM Chang, CH Chen, TY Hou, SY Kuo, JH Lai. A comparison of the diagnostic sensitivity and specificity of two anti-cyclic citrullinated peptides (CCP1 and CCP2) tests for rheumatoid arthritis. J Rheumatol R.O.C. 2006; 20:19-24.
 14. FC Liu, DM Chang, JH Lai, CK Lin, **HC Chen**. Autoimmune hepatitis with raised alpha-fetoprotein level as the presenting symptoms of systemic lupus erythematosus: a case report. Rheumatol Int. 2007; 27:489-91. (SCI)
 15. Hou TY, Chang DM, Lai JH, Chen CH, **Chen HC**, Liu FC, Chang CC. Sonographic findings in rheumatic diseases. Journal of international medicine of Taiwan 2007;18: 332-41.
 16. Hou TY, **Chen HC**, Chen CH, Chang DM, Liu FC, Lai JH. Usefulness of human leucocyte antigen-B27 subtypes in predicting ankylosing spondylitis: Taiwan experience. Intern Med J. 2007; 37:749-52. (SCI)
 17. Liu FC, Chao YC, Hou TY, **Chen HC**, Shyu RY, Hsieh TY, Chen CH, Chang DM, Lai JH. Usefulness of anti-CCP antibodies in patients with hepatitis C virus infection with or without arthritis, rheumatoid factor, or cryoglobulinemia. Clin Rheumatol. 2008; 27:463-7. (SCI)
 18. **HC Chen**, SH Shah, TV Stabler, YJ Li, and VB kraus. Biomarkers associated with clinical phenotypes of hand osteoarthritis in a large multigenerational family: the CARRIAGE Family study. Osteo Cartilage. 2008; 16:1054-9 (SCI).
 19. **HC Chen**, SH Shah, YJ Li, TV Stabler, JM. Jordan, VB Kraus. Inverse association of general joint hypermobility with hand and knee osteoarthritis and serum cartilage oligomeric matrix protein levels. Arthritis Rheum. 2008; 58:3854-64 (SCI).

Papers in preparation

1. **HC Chen***, VB Kraus*, YJ Li, S Nelson, C Haynes, J Johnson, TV Stabler, ER Hauser, SG Gregory, WE Kraus, SH Shah. Genome-wide linkage analysis of quantitative biomarker traits of osteoarthritis: evidence for novel linkage on chromosome 8. (*contributed equally to this work)

2. **HC Chen**, D Thompson, SH Shah, YJ Li, S Nelson, C Haynes, J Johnson, ER Hauser, SG Gregory, WE Kraus, VB Kraus. MMP-3 replicate previous radiographic and biomarkers-related susceptibility loci on chromosome 6. (short report) (in preparation)
3. **HC Chen**, FC Liu, DM Chang. Catastrophic lupus: cavernous sinus thrombosis (image). (in preparation)

Abstracts (USA):

1. **Chen, HC**, T Stabler, and VB Kraus. Biomarkers associated with clinical phenotypes of hand osteoarthritis in a large multigenerational family: CARRIAGE family. Osteoarthritis Cartilage 2007: 15:C70-71. (accepted as a poster)
2. **Chen, HC**, T Stabler, and VB Kraus. 2007. Association of osteoarthritis, osteoarthritis biomarkers and articular hypermobility. Osteoarthritis Cartilage 2007: 15:C47-48. (podium presentation)
3. **Chen HC**, Shah SH, Li YJ, Nelson S, Haynes C, Johnson J, Stabler T, Hauser ER, Gregory SG, Kraus WE, Kraus VB. 2008 Whole Genome Scan in the CARRIAGE Family Study: Evidence of Novel Quantitative Trait Loci (QTL) for Osteoarthritis Trait on Chromosome 8. American Journal of Human Genetics 2008: C2051. (accepted as a poster)
4. **Chen HC**, Li YJ, Kraus KB. Heritability of Cartilage Oligomeric Matrix Protein and General Joint Hypermobility: the CARRIAGE Family Study. 55th annual meeting ORS 2009: Supp 109 (accepted as a poster)