Cartilage-derived biomarkers of osteoarthritis in synovial fluid of dogs with naturally acquired rupture of the cranial cruciate ligament

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Objective—To compare synovial fluid biomarkers of cartilage metabolism in joints with naturally acquired or experimentally induced cranial cruciate ligament (CCL) rupture and determine correlations with stage and severity of disease in dogs.

Animals—95 dogs with ruptured CCL, 8 dogs with experimentally ruptured CCL, and 24 healthy dogs.

Procedures—Synovial fluid was assayed for chondroitin sulfate neo-epitopes 3B3(–) and 7D4 and glycosaminoglycan (GAG) concentration. Results were correlated with demographic data, duration of lameness, radiographic osteoarthritis score, and intra-articular lesions.

Results—The 7D4 concentrations and 7D4:GAG in synovial fluid from joints with naturally acquired CCL rupture and experimental CCL transection were similar and significantly greater than values for healthy control joints. The 3B3(–) concentrations in the CCL-deficient groups were not significantly different, although only values in the naturally acquired CCL rupture group were significantly greater than those in the healthy control group. Within the naturally acquired CCL rupture group there was a significant correlation between 3B3(–) and 7D4 concentrations. However, there were no significant correlations between biomarker concentrations and continuous demographic or disease-related variables or differences in biomarker concentrations with different categories of disease.

Conclusions and Clinical Relevance—Synovial fluid biomarker concentrations were significantly increased in joints with secondary osteoarthritis associated with naturally acquired or experimental CCL rupture; however, lack of apparently simple relationships with demographic variables or stage or severity of disease limits their clinical usefulness. (Am J Vet Res 2002;63:775–781)

Synovial fluid has important functions in joint lubrication and articular cartilage nutrition. A highly viscous modified dialysate of plasma that contains hyaluronan, synovial fluid is secreted from the synovial membrane into the joint cavity. Various intact, cleaved, and partially degraded matrix molecules are also released from articular cartilage into the synovial fluid with ongoing turnover of cartilage extracellular matrix. Normally such molecules leave the joint in lymph or blood to be endocytosed, metabolized in the liver, or excreted in urine. After joint injury and with development of osteoarthritis (OA), the quantity and type of such matrix molecules released from damaged and degraded articular cartilage into the synovial fluid are modified. Presently, a major focus of research is the study of cartilage-specific molecules in synovial fluid as biomarkers of altered articular cartilage metabolism. Synovial fluid sampling by use of arthrocentesis has advantages of being accessible, relatively noninvasive, and repeatable longitudinally. Thus, the hope is that studies of cartilage-specific biomarkers in synovial fluid will improve our understanding of mechanisms involved in OA, facilitate earlier diagnosis of OA before the cartilage has been irreversibly damaged, and allow the effects of treatment to be monitored.

Aggrecan is the major proteoglycan aggregate of articular cartilage, and it is responsible for the compressive stiffness properties of cartilage. In early stage OA there are increases in both degradation and synthesis of aggrecan and other matrix molecules by articular chondrocytes. Cleavage of matrix aggrecan into fragments is thought to occur in the interglobular domain by an aggrecanase and at secondary sites by matrix metalloproteinases, and consequential loss of aggrecan from cartilage matrix into the synovial fluid is one of the critical events in the development of OA (Fig 1). The concentration of aggrecan fragments in synovial fluid is increased in human knee injury and OA, with concentrations being higher in early-stage disease than later. Similarly, increased concentrations of a C-telopeptide cross-linking domain of type-II collagen have been detected in synovial fluid early after knee joint injury, indicating that loss of mature cross-linked type-II collagen from cartilage matrix occurs in conjunction with aggrecan loss.

In early-stage OA, a feature of newly synthesized cartilage aggrecan molecules is that they contain chondroitin sulfates that have increased chain length and altered sulfation patterns and structure, exposing
unique neo-epitopes that are detectable by use of monoclonal antibodies such as 3B3 and 7D4 (Fig 1). It has been proposed that these neo-epitopes are anabolic markers of cartilage turnover during OA, and that they are indicative of attempts by chondrocytes to repair or remodel damaged cartilage. Antibody 3B3 recognizes a neo-epitope on native, nonenzymatically cleaved chondroitin sulfate chains that have a nonreducing termination of GlcAβ1,3GalNAc6S; this neo-epitope is called 3B3(–). Monoclonal antibody 7D4 recognizes a neo-epitope within native chondroitin sulfate chains that is 6-sulfated and contains a single nonsulfated disaccharide. Expression of 7D4 neo-epitope is more prevalent in proteoglycans extracted from osteoarthritic cartilage and synovial fluid from humans, monkeys, and guinea pigs, compared with normal cartilage. Concentrations of 7D4 neo-epitope, but not 3B3(–), are significantly increased in synovial fluid from patients with acute knee joint injury and early traumatically induced OA; the increases are greatest within the first 3 months after trauma. Similarly, another study that evaluated cartilage damage arthroscopically within the first 4 months after knee injury found a negative correlation between synovial fluid 3B3(–) concentration and severity of articular cartilage damage. By contrast, synovial fluid concentrations of 3B3(–), but not 7D4, were significantly increased in human knee joints with chronic OA. These apparently disparate results are an indication that synovial fluid cartilage biomarker concentration may be influenced by many factors, including species, and duration and severity of disease.

There have been few studies in experimental animals to evaluate factors that influence concentrations of synovial fluid biomarkers in OA. One longitudinal study of synovial fluid cartilage biomarkers that used a canine model of OA experimentally induced by use of CCL transection found that an increase in 7D4 concentration preceded the rise in 3B3(–), but both reached a plateau in the period 3 to 5 months after surgery. While these findings differ from observations in humans, we recognize that any particular animal model might replicate only certain aspects of human disease. Therefore it can be fruitful to not only consider studying different species of animals with induced OA as models, but also to evaluate well-defined populations of animals with naturally acquired disease. Thus the purpose of our study was to compare cartilage biomarker concentrations in synovial fluid from dogs with naturally acquired OA with those found in experimentally induced OA. Furthermore, we wanted to study possible correlations of synovial fluid cartilage biomarker concentrations with stage and severity of spontaneous canine OA.

Materials and Methods

Dogs with naturally acquired rupture of the CCL—Ninety-five adult dogs evaluated at the University of Wisconsin-Madison Veterinary Teaching Hospital that had CCL rupture were studied. Mean age was 4.7 years (range, 1 to 10 years), mean body weight was 30.3 kg (range, 15 to 76 kg), and the most commonly represented breeds were Labrador Retriever (38%) and Golden Retriever (17%). Duration of lameness on the affected limb was ascertained from historical information provided by owners. Mediolateral and cranio-caudal radiographic views of affected joints were obtained before surgery. Synovial fluid was collected from the femoropatellar compartment of joints by use of direct arthrocentesis, using a 5-ml plastic syringe and 19- to 21-gauge needle at the time of surgery, just prior to incising the joint capsule and performing an arthrotomy. We attempted to collect the maximum volume of synovial fluid possible, while trying to avoid excessive use of negative pressure and contamination of the synovial fluid sample with blood.

Dogs with experimentally transected CCL—Synovial fluid was collected via arthrocentesis from 1 stifle joint of 8 large, mixed-breed mature male dogs (body weight range, 23 to 32 kg) with OA secondary to experimental CCL transection 13 weeks previously. These dogs were from the control groups to 32 kg) with OA secondary to experimental CCL transection of 2 unrelated studies that had CCL rupture were studied. Mean age was 4.7 years (range, 1 to 10 years), mean body weight was 30.3 kg (range, 15 to 76 kg), and the most commonly represented breeds were Labrador Retriever (38%) and Golden Retriever (17%). Duration of lameness on the affected limb was ascertained from historical information provided by owners. Mediolateral and cranio-caudal radiographic views of affected joints were obtained before surgery. Synovial fluid was collected from the femoropatellar compartment of joints by use of direct arthrocentesis, using a 5-ml plastic syringe and 19- to 21-gauge needle at the time of surgery, just prior to incising the joint capsule and performing an arthrotomy. We attempted to collect the maximum volume of synovial fluid possible, while trying to avoid excessive use of negative pressure and contamination of the synovial fluid sample with blood.

Clinically normal control dogs—Synovial fluid samples were obtained from stifle joints of 24 healthy adult large mixed- and pure-breed dogs that were euthanatized for reasons unrelated to joint disease. Dogs were of unknown age. Body weights were not recorded, and no radiographs were taken. Lack of gross signs of OA or CCL rupture was confirmed by visual inspection at necropsy.

Synovial fluid—Synovial fluid was centrifuged at 12,000 X g for 10 minutes at 4 °C to remove cells, and the supernatant was stored at –80 °C until assayed.

Radiographic grading—Severity of OA in stifle joints of dogs in the naturally acquired OA group was graded on radiographs by use of an established scheme (Appendix) for which total cumulative joint score ranged from 0 (normal) to 60 (end-stage OA). Scoring was performed by 1 investigator (CWH) who was unaware of results of synovial fluid analyses.
Intraoperative joint evaluation—Joint lesions were assigned an intraoperative score by the surgeon, using criteria for the CCL (1 = normal, 2 = partially torn, 3 = completely ruptured with remnants of ligament remaining, 4 = ligament ruptured and completely resorbed), and the medial meniscus (1 = normal, 2 = partial tear, 3 = caudal pole folded or macerated). Cartilage and synovial membrane lesions observed at surgery were also scored and recorded, but because we subsequently found that the criteria used for grading could not be objectively applied in a reproducible manner, these data were not further analyzed.

Sulfated glycosaminoglycan (GAG) content of synovial fluid—Sulfated GAG concentrations were measured by use of a dye-binding assay, as described.87 Shark chondroitin sulfatase standards in the range of 0 to 40 μg/ml were prepared. After addition of 1.9 dimethylmethylene blue (DMMB) solution13 to each plate well containing sample or standard, shifts in absorbency were detected immediately at 530 nm on a plate reader.

Western blot analysis—In a subset of dogs (31 joints with naturally acquired rupture of the CCL and 5 clinically normal control joints) for which there was sufficient volume of sample, synovial fluid was separated via gel electrophoresis and immunoblotting was performed with 3B3 and 7D4 monoclonal antibody as follows. Synovial fluid samples were suspended in equal volumes of 100 mM sodium acetate buffer, pH 6.8, containing 0.2M 6-amino hexanoic acid, 10 mM benzamidine HCl, 20 mM EDTA, and 2 mM phenyl methyl sulphonyl fluoride. Samples were separated on associative CsCl (1.50 g/ml) density gradients by centrifugation for 36 hours at 100,000 g in a TLA 100.3 rotor (Beckman). After addition of 1,9 dimethylmethylene blue (DMMB) solution13 to each plate well containing sample or standard, shifts in absorbency were detected immediately at 530 nm on a plate reader.

Results

Western blot immunostaining of synovial fluid with monoclonal antibody 3B3 was confined to a sin-
gle, slow-migrating large proteoglycan subpopulation, whereas 7D4 immunostaining was seen in several faster-migrating proteoglycan subpopulations. Staining intensity for both neo-epitopes was significantly greater in synovial fluid from joints with naturally acquired CCL rupture than in clinically normal joints (Fig 2).

Analysis of ELISA data by use of the Kruskal-Wallis test found significant overall differences among groups for 3B3(–), 7D4, and 7D4/GAG; differences among groups for GAG approached significance (P = 0.054; Table 1). Investigation of these differences in the 3B3(–), 7D4, and 7D4/GAG data by use of post-hoc Wilcoxon ranks sums test found that concentrations of 7D4 and 7D4/GAG were similar in synovial fluid of joints with naturally acquired CCL rupture and experimentally transected CCL, and that concentrations in both groups were significantly greater than values for clinically normal joints (Table 1). Although 3B3(–) concentrations for both the CCL deficient groups were similar, it was only in the naturally acquired CCL rupture group that the 3B3(–) concentration was significantly greater than the clinically normal control group. For 3B3(–), the difference between the experimentally transected CCL and clinically normal control groups approached significance (P = 0.024).

None of the continuous demographic or disease-related variables had significant correlation with the biomarker concentrations (Table 2). However, there

### Table 1—Synovial fluid cartilage biomarkers (3B3[–]), 7D4, and glycosaminoglycan (GAG); median and interquartile values from joints of clinically normal control dogs and dogs with naturally acquired cranial cruciate ligament (CCL) rupture and experimental CCL transection

<table>
<thead>
<tr>
<th>Variable</th>
<th>Natural acquired CCL rupture</th>
<th>Experimental (n = 5–9)</th>
<th>Control (n = 18–26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3B3(–) (ng/ml)</td>
<td>(100–191)</td>
<td>164.4 (153.9)</td>
<td>110.8 (95.6)</td>
</tr>
<tr>
<td>7D4 (µg/ml)</td>
<td>(6–83.2)</td>
<td>67.2 (65.3)</td>
<td>11.6 (4.8)</td>
</tr>
<tr>
<td>3B3(–)/GAG (ng/µg)</td>
<td>(1.3–3.9)</td>
<td>2.5 (2.5)</td>
<td>2.5 (2.5)</td>
</tr>
<tr>
<td>7D4/GAG (µg/µg)</td>
<td>(0.7–1.2)</td>
<td>1.1 (0.9)</td>
<td>0.9 (0.8)</td>
</tr>
</tbody>
</table>

*P values derived from Kruskal-Wallis test for overall differences among the 3 groups. Values of P < 0.05 were considered significant.

Within each row, values with different superscripts are significantly different (after Bonferroni correction).

### Table 2—Evaluation of correlations (Spearman correlation P value [P value for test of correlation] between synovial fluid biomarker (3B3[–] and 7D4) concentrations and demographic and disease-related variables in dogs with naturally acquired rupture of the CCL

<table>
<thead>
<tr>
<th>Variable</th>
<th>3B3(–)</th>
<th>7D4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.071 (0.493)</td>
<td>0.027 (0.795)</td>
</tr>
<tr>
<td>Body weight</td>
<td>-0.039 (0.578)</td>
<td>-0.033 (0.754)</td>
</tr>
<tr>
<td>Duration</td>
<td>0.060 (0.563)</td>
<td>-0.065 (0.538)</td>
</tr>
<tr>
<td>Radiographic score</td>
<td>-0.087 (0.402)</td>
<td>-0.144 (0.167)</td>
</tr>
<tr>
<td>CCL score</td>
<td>0.109 (0.102)</td>
<td>0.085 (0.115)</td>
</tr>
<tr>
<td>Medial meniscus score</td>
<td>0.097 (0.351)</td>
<td>0.049 (0.844)</td>
</tr>
</tbody>
</table>

was a significant Spearman correlation (0.3973) between 3B3(–) and 7D4 (P value for independence, < 0.001). There were no significant differences in concentrations of 3B3(–) and 7D4 between the categoric demographic (sex and limb) or disease-related variables (CCL and medial meniscus scores).

### Discussion

Rupture of the CCL in dogs is not usually an acute traumatic event, and it is more often associated with progressive chondroid degenerative changes within the ligament, which in turn leads to weakening and subsequent partial or complete failure.24-26 Associated with these ligamentous changes are concurrent degeneration of articular cartilage and menisci, synovitis, and osteophytosis.27,28 Although such degeneration of the anterior cruciate ligament in humans is also recognized, most such ruptures in humans are the result of acute loading with trauma and sports-related injuries. Our results indicate that naturally acquired CCL rupture in dogs is associated with significant increases in 3B3(–) and 7D4 epitopes in synovial fluid. Furthermore, despite the differences in pathogenesis of naturally acquired CCL rupture and experimental CCL transection in these canine models, the alterations in synovial fluid biomarkers in these 2 groups were essentially similar.

In a previous study, transection of the canine CCL resulted in an increase in synovial fluid 7D4 concentration that preceded the rise in 3B3(–), but both reached a plateau after several months.19 These changes were consistent with initiation and progression of OA, and were not significantly influenced by either intra-articular or extracapsular reconstruction of the transected CCL.19 Two other preliminary reports30 described studies of 3B3 and 7D4 epitopes in articular cartilage after CCL transection and reconstruction in dogs. Densitometric analysis of immunoblots of cartilage for 3B3(–) and 7D4 revealed that expression of epitope in articular cartilage after 28 weeks was similar in CCL-transected joints after a sham reconstruction and intra-articular reconstruction using the medial one third of the patellar tendon.1 However, results of synovial fluid assays for 3B3(–) and 7D4 were unrewarding, and concentrations did not correlate with development of OA.1 Perhaps this may have related to collection of synovial fluid by use of joint lavage with saline solution because the final concentration of epitope could not be accurately calculated, because the initial volume of synovial fluid in the joint was not determined.

Quantification of 3B3(–) as a biomarker on cartilage aggrecan metabolites has been questioned in chemical and immunologic assays of the non reducing terminal residues of chondroitin sulfate from aggrecan of clinically normal and osteoarthritic human joints.12,29 However, it should be pointed out that the immunologic detection of 3B3(–) epitope and the chemical detection of its non reducing structure are quite different analytic measurements. Immunologic detection of 3B3(–) epitope on chondroitin sulfate chains depends on the spatial presentation and availability of these epitopes to the 3B3 IgM antibody, whereas chemical detec-
ticular cartilage lesions. Results of studies15-17 of there is not a simple linear relationship between con-
articular cartilage metabolism during OA, and that biomarkers presently only provide evidence of altered
ied independently of each other. This disparity high-
losing may provide a more sensitive in vivo assessment of information about articular cartilage damage. These are scores based on this imaging modality provide minimal
sion, osteophytes, and subchondral bone remodeling; Furthermore, radiology can only reliably detect effu-
the pathologic processes in affected joints in these dogs.

Although we found good correlation between 3B3(–) and 7D4 epitope concentrations in synovial fluid from dogs with naturally acquired rupture of the CCL, another study15 of human knee joint synovial fluid found that concentrations of these 2 epitopes varied independently of each other. This disparity highlights the importance of appreciating that synovial fluid biomarkers presently only provide evidence of altered articular cartilage metabolism during OA, and that there is not a simple linear relationship between concentration of any particular biomarker and severity of articular cartilage lesions. Results of studies3,7,8,11,13-16,30-32 clearly indicate that by using immunologic detection and quantification of 3B3(–) epitope (ie, Western blot, ELISA, or both), measurement of this epitope can be an indicator of changes in aggrecan metabolism with the onset of cartilage damage. Since injury, and chronicity of OA.Perhaps there are species differences as well. Contrary to what we were expecting, we did not detect any significant correlations between biomarker concentration and any of the demo-
graphic or disease-related variables in the dogs with naturally acquired CCL rupture results in significant
which such changes may not become an important problem for 1 or 2 decades.36-38 Our results indicate that naturally acquired CCL rupture results in significant increases in concentrations of 3B3(–) and 7D4 epitopes in synovial fluid, compared with normal canine synovial fluid. These changes were similar to those observed after experimental transection of the canine CCL. Thus, this naturally acquired disease of the canine CCL represents an opportunity to use this as a model for minimally invasive studies of OA by use of techniques such as syn-

Differences between concentrations of GAG in synovial fluid in joints of dogs of the 2 groups with
ruptured CCL (naturally or experimentally) and those of clinically normal control dogs approached signifi-
cance. In previous studies16,13,40 of dogs with experimental CCL transection and naturally acquired CCL
rupture, GAG concentrations in synovial fluid were not increased, compared with contralateral normal joints or samples taken from clinically normal dogs. Furthermore, GAG concentrations were not correlated with age, sex, body weight, disease duration, or CCL reconstruction.16,39 In attempting to interpret data for GAG concentrations and other biomarkers in synovial fluid, we should consider rate of release from articular cartilage, total volume of joint effusion at the time of collection, and rate of removal of fragments and GAG from the synovial fluid. One of the ongoing controvers-
vies in synovial fluid biomarker studies is the influ-
ence of changes in synovial fluid volume in joints with effusion and the consequent dilution of biomarkers. In attempting to control these factors, we chose to also express epitope concentration as a ratio of GAG concentration, as had been done previously.13,41

Lohmander LS, Atley LM, Pietka TA, et al. The development of osteoarthri-

Ong-Chai S. Invesigation of the epitope in chondroitin sulphate recog-
nised by the monoclonal antibody 7D4. PhD thesis. School of Biological Sciences, University of Manchester, Manchester, UK, 1999.

Chondroitin sulphate c sodium salt, Sigma Chemical Co, St Louis,


Monoclonal antibody 3B3, Seikagaku Corp, Tokyo, Japan.

Proto-blot Western Blot AP systems kit, Promega, Madison, Wis.

Provided by Professor Michael T. Bayliss, Royal Veterinary College, University of London, London, UK.

Anti-mouse IgM peroxidase conjugate, Sigma Chemical Co, St Louis, Mo.

Peroxidase substrate ABTS, Sigma Chemical Co, St Louis, Mo.


Wang H, Dahners L, Roe S, et al. The development of osteoarthri-
Appendix
Criteria used for radiographic grading of stifle joint osteoarthritis in dogs (modified from Vasseur and Berry 

<table>
<thead>
<tr>
<th>Compartment and Features</th>
<th>Features</th>
</tr>
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<tbody>
<tr>
<td>Femoropatellar</td>
<td>Apical or basilar patellar osteoarthropathies in cases with arthritis?</td>
</tr>
<tr>
<td>Apical or basilar patellar osteoarthropathies in cases with arthritis?</td>
<td></td>
</tr>
<tr>
<td>Femoral trochlear groove osteoarthropathies in cases with arthritis?</td>
<td></td>
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<tr>
<td>Femoral supratrochlear lysis</td>
<td></td>
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<tr>
<td>Lateral femoral</td>
<td></td>
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<tr>
<td>Femoral and tibial paticular osteoarthropathies in cases with arthritis?</td>
<td></td>
</tr>
<tr>
<td>Femoral and tibial subchondral sclerosis in cases with arthritis?</td>
<td></td>
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<tr>
<td>Femoral and tibial remodeling in cases with arthritis?</td>
<td></td>
</tr>
<tr>
<td>Subchondral lysis of femur and tibia in cases with arthritis?</td>
<td></td>
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<tr>
<td>Lateral fabellar osteoarthropathies in cases with arthritis?</td>
<td></td>
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<tr>
<td>Lateral collateral ligament osteoarthropathies in cases with arthritis?</td>
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<tr>
<td>Medial femoral</td>
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<tr>
<td>Femoral and tibial paticular osteoarthropathies in cases with arthritis?</td>
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<td>Medial fabellar osteoarthropathies in cases with arthritis?</td>
<td></td>
</tr>
<tr>
<td>Medial collateral ligament osteoarthropathies in cases with arthritis?</td>
<td></td>
</tr>
<tr>
<td>Central femoral</td>
<td></td>
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<tr>
<td>Cranial or caudal osteoarthropathies on tibia in cases with arthritis?</td>
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</tr>
<tr>
<td>Femoral intercondylar notch width in cases with arthritis?</td>
<td></td>
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<tr>
<td>Intercondylar avulsion fracture fragments in cases with arthritis?</td>
<td></td>
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<tr>
<td>Tibial subchondral sclerosis in cases with arthritis?</td>
<td></td>
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</tbody>
</table>

Each feature was assigned a value of 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). Scores were added to produce a cumulative joint score ranging from 0 to 60.

References


