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Link to publication

Tenascin-C levels in synovial fluid are elevated after injury to the human and canine joint and correlate with markers of inflammation and matrix degradation

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Abstract

Objective - We have previously shown the capacity of tenascin-C (TN-C) to induce inflammatory mediators and matrix degradation *in vitro* in human articular cartilage. The objective of the present study was to follow TN-C release into knee synovial fluid after acute joint injury or in joint disease, and to correlate TN-C levels with markers of cartilage matrix degradation and inflammation.

Method - Human knee synovial fluid samples (n=164) were from a cross-sectional convenience cohort. Diagnostic groups were knee healthy reference, knee anterior cruciate ligament rupture, with or without concomitant meniscus lesions, isolated knee meniscus injury, acute inflammatory arthritis and knee osteoarthritis. TN-C was measured in synovial fluid samples using an ELISA and results correlated to other cartilage markers. TN-C release was also monitored in joints of dogs that underwent knee instability surgery.

Results – Statistically significantly higher levels of TN-C compared to reference subjects were observed in the joint fluid of all human disease groups and in the dogs that underwent knee instability surgery. Statistically significant correlations were observed between the TN-C levels in the synovial fluid of the human patients and the levels of aggrecanase-dependent ARG-aggrecan fragments and matrix metalloproteinases 1 and 3.

Conclusions - We find highly elevated levels of TN-C in human knee joints after injury, acute inflammatory arthritis or osteoarthritis that correlated with markers of cartilage degradation and inflammation. TN-C in synovial fluid may serve dual roles as a marker of joint damage and a stimulant of further joint degradation.

Key words: Tenascin-C, joint injury, synovial fluid, biomarker, cartilage

Running title: Joint injury and elevated Tenascin-C levels

Introduction

Tenascin-C (TN-C) is a six-armed extracellular matrix glycoprotein initially discovered at the myotendinous junction[1,2]. It is a member of a family of related proteins comprising TN-C, -R, -X, and –Y[1-3]. Tenascins have a modular composition with an amino-terminal oligomerization domain consisting of heptad repeats, multiple epidermal growth factor (EGF)-like repeats, a variable number (from 8 to 14) of fibronectin type III modules, and a carboxyl-terminal fibrinogen-like globular domain[4]. The heptad repeat sequences facilitate the formation of hexamers, in which form TN-C is deposited in the tissues[1,2]. It interacts with other ECM molecules such as integrins, collagens, proteoglycans, heparin, syndecan, and fibronectin. It plays a major role in cell adhesion, migration, proliferation, and cellular signaling[5]. In addition, TN-C acts as an elastic protein that can stretch to several times its resting length by stretch-induced unfolding of its fibronectin type III domains[6].

TN-C is abundantly expressed in musculoskeletal tissues during organogenesis and embryogenesis, while its expression is very restricted in healthy tissues, and reappears as a high molecular weight splice variant in association with wound healing, inflammatory processes, or neoplasia[7,8]. TN-C is associated with the development of articular cartilage, but decreases markedly during maturation of chondrocytes[9,10], and almost disappears in adult cartilage[11,12]. TN-C is highly re-expressed in the cartilage and the synovium of diseased or injured joints[11-15]. There is a correlation between the levels of TN-C in joint fluids and degree of cartilage degradation[16] or radiographic severity of OA[17]. It stimulates the production of proinflammatory cytokines in human macrophages and synovial fibroblasts through activation of Toll-like receptor-4 (TLR4)[18].

We have shown elevated levels of TN-C in joint fluid in animal models of joint disease, and demonstrated the capacity of TN-C to induce inflammatory mediators and matrix degradation *in vitro* in human joint cartilage[19]. The objective of the present study was to determine the levels of TN-C in synovial fluid of patients with joint injury and joint disease, and to correlate levels of TN-C with markers of cartilage matrix degradation and inflammation in the same fluids. TN-C levels were also examined in synovial fluids of a dog model of joint injury.

Methods

Human synovial fluid samples

Human knee synovial fluids (n=164) were aspirated without lavage from a cross-sectional convenience cohort with informed consent and approval of the Lund University Research Ethics Committee. Diagnosis was made by arthroscopy, radiography, assessment of joint fluid and clinical examination. Diagnostic groups were knee healthy references without current or past knee symptoms or injury (REF), acute inflammatory arthritis (pseudogout, pyrophosphate crystal arthritis, AIA), knee anterior cruciate ligament rupture with or without concomitant meniscus lesions (ACL), isolated knee meniscus lesions (MEN), and knee osteoarthritis (OA) (*Table 1*). The OA grades of these groups were based on combined arthroscopic and radiographic data as described[20], where grade 1 represents no structural change, 2-5 represent increasing severity and extent of arthroscopically observed cartilage damage, and 6-10 represent increasing severity of radiographic changes. Patient availability determined the number of samples within each group, as well as age and sex distribution within each diagnostic group. The volume of joint fluid available from each patient allowed only a limited and variable number of different biomarker assays for each sample.

Dog knee joint instability model

Instability surgery was performed in 9.6 (9.1 - 9.9 range) month old skeletally mature, purpose-bred male cross-bred hounds (Marshall Farms, New Rose NY) weighing 19.4 kg (17.1 - 21.9 kg range) in Massachusetts and Pearl River Pfizer (formerly Wyeth) Institutional Animal Care and Use Committee (IACUC)-approved protocols. Dogs were anesthetized with isoflurane and pre-operative radiographs obtained to ensure that no radiographic joint abnormalities were present before instability induction. Fentanyl patches were applied pre-operatively and kept in place for 3 days to provide sustained post-operative pain relief. Anterior cruciate ligament (ACLT) is an established instability model of OA induction in dogs[21,22]. A 5 cm longitudinal incision was made laterally in the femoro-tibial joint, and an arthroscopic knife (with a retrograde blade) used to transect the ACL under direct visualization. Strict attention was paid to hemostasis. The transection was confirmed by presence of anterior drawer, and copiously flushed with saline. The joint was closed in 4 layers (synovium, joint capsule, subcutaneous and skin). Surgery on the contralateral knee involved sham surgery that consisted of opening of the joint without ACLT. Dogs had cage

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rest for 10 days after surgery. At 1, 3 and 6 months post-surgery, 4-5 animals per time point were randomly selected and euthanized via acepromazine sedation and pentobarbital overdose. The knee joint fluids were aspirated from both ACLT and contralateral sham knees. Cartilage was harvested for other studies.

Tenascin-C immunoassay

TN-C was measured using TN-C Large ELISA (IBL® http://www.ibl-japan.co.jp), with anti-TN-C 19C4MS monoclonal antibody against the FNIII-C domain for capture, and HRP-conjugated anti-TN-C 4F10TT mouse monoclonal antibody against the EGF-like domain for detection. Monoclonal antibody 19C4MS binds an epitope of the FNIII-C domain and recognizes large TN-C variants, while 4F10TT binds an epitope from the EGF domain and recognizes both the small and large TN-C variants. Details of the assay and antibodies have been published[17,19,23]. Samples were diluted in PBS before being assayed following the manufacturer's protocol.

Other biomarkers and assays

The human joint fluid samples assayed for TN-C in the present study were obtained in the course of previous studies, and assay results for the F21-epitope of aggrecan, 846 epitope of aggrecan, cartilage oligomeric matrix protein (COMP), type II collagen cross-linked C-telopeptide fragments (CTX-II), procollagen II C-propeptide (PCIIC), matrix metalloproteinase (MMP) 1, MMP-3, tissue inhibitor of matrix metalloproteinases 1 (TIMP-1), MMP-1-activity, and MMP-3-activity were in part published in the course of these studies[24-32]. Immunoassays were performed as described for ARG-neoepitope aggrecan fragments[24,25], aggrecan epitopes using the 1-F21 and the 846 monoclonal antibodies[24,26], COMP, CTX-II, PCIIC, MMP-1, MMP-3, and TIMP-1[27-30]. Synovial fluid MMP-1 and -3 proteinase activities were assayed as described[32].

Statistical methods

Data were analyzed using Sigmaplot 12 and STATA 12.1. Biomarker concentrations were not normally distributed and were therefore logarithmically transformed for statistical analysis, following which values were normally distributed by Shapiro-Wilk's test, allowing parametric

tests for diagnostic group differences by one-way ANOVA with Holm-Sidak correction for multiple comparisons against the reference group. The influence of OA grade, gender, age and sampling time after injury or onset of symptoms on TN-C concentrations in human diagnostic groups was analyzed by multiple linear regression analysis. To explore the influence of time between injury and sampling on TN-C concentration in synovial fluids, the diagnostic groups ACL and MEN were merged, and samples ordered by time windows as in Fig. 2. Time-related statistical differences were assessed by ANOVA. Correlations between concentrations of different synovial fluid biomarkers were determined by Pearson's method. The crude mean fold change in TN-C among the patient groups ACL, AIA, MEN, and OA, versus the mean value of the REF group, was estimated and statistically tested with logarithmically transformed values using a linear regression model (ANOVA). Adjustment for differences in age and sex was achieved by including these variables as covariates in the linear model (ANCOVA). The presented results, in terms of fold change and 95% confidence interval limits, were re-transformed back to the original scale. The fulfillment of the assumption that the model residual had a Gaussian distribution assumption was evaluated graphically using a normal probability plot. The mean fold change in TN-C among dogs was estimated with 95% confidence interval using logarithmically transformed within-dog fold change. It was also tested, against the null hypothesis that ln(fold change) is nil, using the one-sample t-test. The presented results, in terms of fold change and 95% confidence interval limits, were re-transformed back to the original scale. The fulfillment of the assumption that within-dog TN-C fold change had a Gaussian distribution was evaluated graphically using a normal probability plot. Statistical significance was set at 0.05.

Results

TN-C levels in human synovial fluids varied widely between 44 and 2430ng/ml. Levels of synovial fluid TN-C were statistically significantly higher in all joint disease and injury groups compared with the REF group (p<0.001, *Figure 1*). Concentrations in the AIA, ACL, MEN and OA groups were on average fold increased over the mean reference group value by 10.9 (95% confidence intervals 5.3, 22.5), 5.0 (2.7, 9.1), 3.5 (2.8, 6.4) and 6.2 (2.8, 14.0), respectively. When the influence on TN-C levels of age, gender, OA-grade and time between synovial fluid sampling and injury or onset of symptoms was examined for the full sample set (n=164), only time between sampling and injury or onset of symptoms showed a statistically

significant relationship with TN-C concentrations in synovial fluid (p=0.004). Accordingly, after injury to the anterior cruciate ligament, meniscus or both, TN-C levels increased markedly in the acute phase, with a maximum of an almost 7-fold increase over the mean reference level between 0 and 12 weeks in the ACL group. With increasing time after joint injury concentrations decreased, but were statistically significantly increased over the REF group at all time windows studied, even beyond one year after injury (p<0.001, *Figure 2*).

At 6 months following ACLT surgery, the operated dog joints showed both macroscopic and histological signs of early stage osteoarthritis in the ACL-deficient joint (*Figure 3*). Similar to the injured human knee joints, TN-C was also increased at all time points in the synovial fluid of the knee of the dogs that underwent ACLT surgery when compared to the fluids of the contralateral sham operated left knee (*Table 2*). In parallel with the human ACL or meniscus injured knees, the dog ACLT knees showed a 68-fold (31.8, 146.1) increase of TN-C at 1 month after ACL transection that declined to around 52- and 49-fold increases at 3 and 6 months after surgery as compared to the contralateral sham-operated knees.

Synovial fluid levels of TN-C in the human samples were statistically significantly correlated with several biomarkers proposed to reflect joint tissue turnover (*Table 3*). Notably, TN-C levels were well correlated with levels of aggrecanase-dependent ARG-aggrecan fragments and type II collagen cross-linked C-telopeptides, less well with aggrecan epitope F21 (lacking specificity for aggrecan proteolytic fragments), but not at all with COMP or PCIIC. Further correlations were noted with the 846 aggrecan epitope, and with protein levels of MMP-1, MMP-3 and TIMP-1, as well as with activity levels of MMP-1 and MMP-3 proteinases.

Discussion

We have found highly increased levels of TN-C in joint fluids following knee injury in humans and in an analogous dog joint injury model, as well as in samples of joint fluid from patients with acute joint inflammation or osteoarthritis. The TN-C levels correlated with several synovial fluid biomarkers that are suggested to reflect joint tissue degradation and repair.

Our results confirm and markedly extend previous reports on the presence of TN-C in human synovial fluids from patients with OA and rheumatoid arthritis[16,17,33]. We found the highest average TN-C concentrations in samples from patients with acute inflammatory arthritis, and note that following human knee joint injury, levels were 7-fold increased in the acute injury phase and more than 3-fold increased even at times longer than 1 year after injury, compared with the reference group. Similar to human joint injury, TN-C levels in dog synovial fluids were greatly increased shortly after injury, and remained elevated for at least 6 months following the joint insult.

Our results are also consistent with previous work showing an increased expression of TN-C in human and animal OA cartilage[19,34], and an increased presence of TN-C in damaged and OA cartilage determined by immunohistochemistry[11-14,35,36].

The cell or tissue source of the TN-C in human or dog synovial fluids in the present study is uncertain, but a local contribution by chondrocytes and synovial cells of the injured joint is likely, given that previous work has documented the production of TN-C by these cells and in cartilage explants when stimulated by proinflammatory cytokines[14,18,19]. The most marked expression increases related to dedifferentiation of chondrocytes were shown for TN-C, type I collagen and hypoxia-inducible factor 1-alpha (HIF-1α)[37]. This does not exclude a systemic contribution to the synovial fluid levels of TN-C. Large (350 and 240kD) and small (210 kD) variants of TN-C have been detected by us and others in human OA synovial fluids[17,19,33], but information on the detailed structure or the mechanism of release of TN-C and TN-C fragments into synovial fluid is lacking. We have earlier reported that both large and small variants were abundant in OA cartilage and synovial fluids when compared to non-OA samples; we found fragments of TN-C of molecular weight lower than 200 kD only in OA cartilage and synovial fluid[19]. Our findings in OA cartilage were later confirmed by the finding that smaller fragments <200 kD are specific to diseased tissue and induce aggrecanase activity[36]. These specific fragments could be MMP generated as large variants are known to be more susceptible to MMP cleavage such as MMP-2 and MMP-7[38]. Considering the low content of TN-C in normal adult articular cartilage, a significant part of the marked increase of TN-C in joint fluid after injury probably represents the product of an upregulated TN-C

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expression by chondrocytes and synoviocytes. The marked increases of synovial fluid TN-C levels found both after dog and human joint injury and in human OA are thus consistent with, and may serve as a marker of a local upregulation of inflammation pathways, and possibly a dedifferentiation of chondrocytes, in these conditions.

The Toll-like receptor-4 (TLR4) is present on human chondrocytes and the TLR4 expression increases with increasing degree of OA cartilage damage[39]. Interestingly, it was shown that TN-C is an endogenous activator of TLR4 and that mice lacking TLR4 are protected from erosive arthritis in a model of inflammatory arthritis[18]. Recent evidence further suggests that synovial dendritic cells may be activated by TN-C, driving IL-17 synthesis in a murine arthritis model[40]. Exposure to exogenous TN-C induced expression of proinflammatory cytokines in synovial explants, macrophages and synoviocytes from individuals with rheumatoid arthritis via activation of TLR4, and elicited dose-dependent joint inflammation in mice[18]. Thus, TN-C fulfills the criteria for an endogenous proinflammatory molecule generated in damaged tissue (damage associated molecular patterns, DAMPs), which through activation of TLR4 mediates persistent inflammation and tissue destruction[18,41]. Consistent with these findings, recent studies report that TN-C fragments, but not full length TN-C, induce expression of ADAMTS4 and a dose dependent release of aggrecanase-generated ARG-aggrecan fragments from the matrix in OA cartilage[36].

The findings briefly summarized here on the role of TN-C through activation of TLR4, with a focus on inflammatory joint disease, suggest that the high concentrations of TN-C we have detected being released into human joint fluids after joint injury and in OA may serve to induce and prolong inflammation and matrix degradation also in these conditions. Activation of TLR through DAMPs such as TN-C may be particularly relevant in the acute phase after joint injury. The correlations noted in this study between TN-C on the one hand, and aggrecanase-generated ARG-aggrecan fragments, type II collagen C-telopeptides, and MMP protein content and protease activity on the other hand, add further support to this hypothesis. We have in the present study not demonstrated a direct relationship between TN-C fragment generation and aggrecanase or MMP activity. However, large variants of TN-C have been shown to be susceptible to MMP cleavage[38], and human cartilage subjected to digestion with aggrecanase generates cleaved TN-C peptides that map to the N-terminal assembly domain,

suggesting that the generation of TN-C fragments by proteases may stimulate proteases and in turn a positive feedback loop of further cartilage matrix degradation[42].

Our study has some limitations, notably in the use of a cross-sectional sample collection of human and canine samples to illustrate time-related events after joint injury. We further acknowledge that validation of the TN-C assay is limited with regard to its specificity and the TN-C variants recognized. In the dog study, we used the contra-lateral knee of the same animal as a control. The bias associated with the use of a 'contra-lateral control joint' is difficult to predict. Firstly, the contra-lateral control joint may be systemically affected by the surgical procedure on the index joint, and may also be affected by changes in joint loading due to the defective index joint. The resulting bias on any 'between-sides' differences are difficult to predict but could serve to decrease apparent differences between the index and control joints. We have interpreted biomarker results based on concentrations in synovial fluid, without consideration of likely variations in synovial fluid and biomarker clearance between different diagnostic groups and different disease phases. As clearance increases with joint inflammation, biomarker concentrations are confounded with regard to their relationship with actual joint tissue biomarker release[43].

In conclusion, TN-C fragments released into synovial fluid after joint injury and in OA may serve as a biomarker of inflammation and chondrocyte dedifferentiation, and at the same time act to induce and prolong inflammation in these conditions. However, intact TN-C in the cartilage appears to have a role in matrix formation, development and repair, and interacts with the aggrecan G3 domain[9,10,44-45], and deficiency of TN-C in knock-out mice is associated with accelerated development of OA and delayed repair of cartilage damage[46]. Further work is needed to fully understand the consequences of the up-regulated synthesis of TN-C, its fragmentation and release into joint fluid after joint injury and in osteoarthritis.

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Figure legends

Figure 1. Box plot of concentrations of TN-C in human synovial fluids from a knee-healthy reference group (REF n=8), and patients with acute inflammatory arthritis (AIA n=18), rupture of anterior cruciate ligament with or without concomitant meniscus lesion (ACL n=57), isolated rupture of the meniscus (MEN n=69), or knee osteoarthritis (OA n=12). Disease groups were all significantly increased over REF, p<0.001. Horizontal line of box indicates median value, box 25th and 75th percentiles, whiskers 10th and 90th percentiles, and symbols outliers.

Figure 2. Box plot of concentrations of TN-C in human synovial fluids from the reference group (REF n=8), and patients with injury to anterior cruciate ligament and/or the meniscus (ACL and MEN combined, n=126). Injury groups were significantly increased over the reference group in all four time windows after injury, p<0.001 (n=37, 19, 29, and 41, respectively). Horizontal line of box indicates median value, box 25th and 75th percentiles, whiskers 10th and 90th percentiles, and symbols outliers.

Figure 3. Representative images of the dog medial femoral condyle at 6 months following ACLT surgery. Top panels show macro views, bottom panels histological sections stained with safranin-O and fast green. Left panels show sham operated contra-lateral joint, right panels show ACLT operated joint.

Table 1
Details of patients and volunteers that provided synovial fluid samples

	REF	AIA	ACL	MEN	OA
n	8	18	57	69	12
Age, mean (range)	28 (17-42)	68 (30-91)	29 (14-53)	38 (16-70)	69 (39-89)
% male	75	63	64	85	45
OA grade, mean (range)	-	6 (1-9)	2 (1-6)	2 (1-8)	8 (3-9)
Sampling time [weeks] after onset or injury, mean (range)	-	29 (0-510)	112 (0-1115)	130 (0-1926)	189 (0-481)

REF = knee healthy references; ACL = knee anterior cruciate ligament rupture; MEN = knee meniscus injury; AIA = acute inflammatory arthritis; and OA = knee osteoarthritis. OA grade 1-10 was assessed as published[20].

Table 2

					TN-C protein in dog knee synovial fluid			
Species	**Category**	**n**	**Mean TN-C in ng/ml**	**Range in ng/ml**	**95% confidence intervals**	**P-value**	**Fold increase over ContL**	**95% confidence intervals for fold increase**
Dog	1m ACLT	4	2817.6	518.3 - 4011.2	1242, 4394	<0.001	68.1	31.8, 146.1
	1m ContL	4	38.7	11.6 - 65.5	16, 61			
	3m ACLT	5	850.3	639.3 - 1261.5	632, 1069	<0.001	52.1	26.6, 102.0
	3m ContL	5	17.8	8.0 - 33.9	9, 26			
	6m ACLT	5	809.1	693.4 - 1113.2	658, 961	<0.001	48.7	38.7, 61.4
	6m ContL	5	16.4	15.1 - 16.8	16, 17			

ACLT: anterior cruciate ligament transection surgery knee, ContL: contralateral sham operated knee, m: month.
Mean TN-C is the mean of the average TN-C values for subjects/animals within a group tested in triplicates.

Table 3
Correlations of different biomarkers with TN-C levels in human synovial fluid samples

	Pearson product moment correlation coefficient of ln transformed values	p	n
ARG-aggrecan	0.462	<0.001	157
F-21 aggrecan	0.251	<0.001	159
846 aggrecan	0.342	<0.001	149
COMP	0.024	0.841	75
CTX-II	0.516	<0.001	137
PCIIC	0.109	0.204	138
MMP-1 protein	0.513	<0.001	152
MMP-3 protein	0.456	<0.001	156
TIMP-1 protein	0.409	<0.001	158
MMP-1 activity	0.474	<0.001	128
MMP-3 activity	0.458	<0.001	140

Abbreviations: ARG-aggrecan, ARG-neoepitope of aggrecanase cleaved aggrecan; F-21 aggrecan, F-21 monoconal antibody epitope of aggrecan; 846 aggrecan, 846 epitope of aggrecan; COMP, cartilage oligomeric matrix protein; CTX-II, type II collagen crosslinked C-telopeptides; PCIIC, type II collagen C-propeptide; MMP-1, -3, matrix metalloproteinase, TIMP-1, tissue inhibitor of matrix metalloproteinase-1. Variable numbers of assay data are a consequence of variable limitations in sample volume.
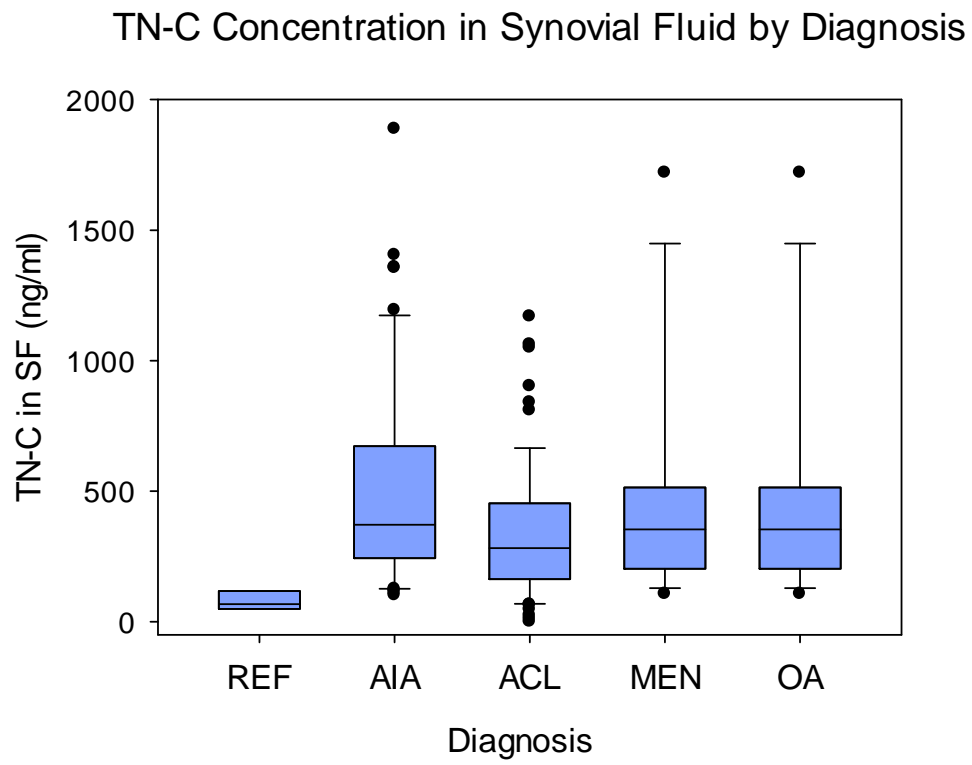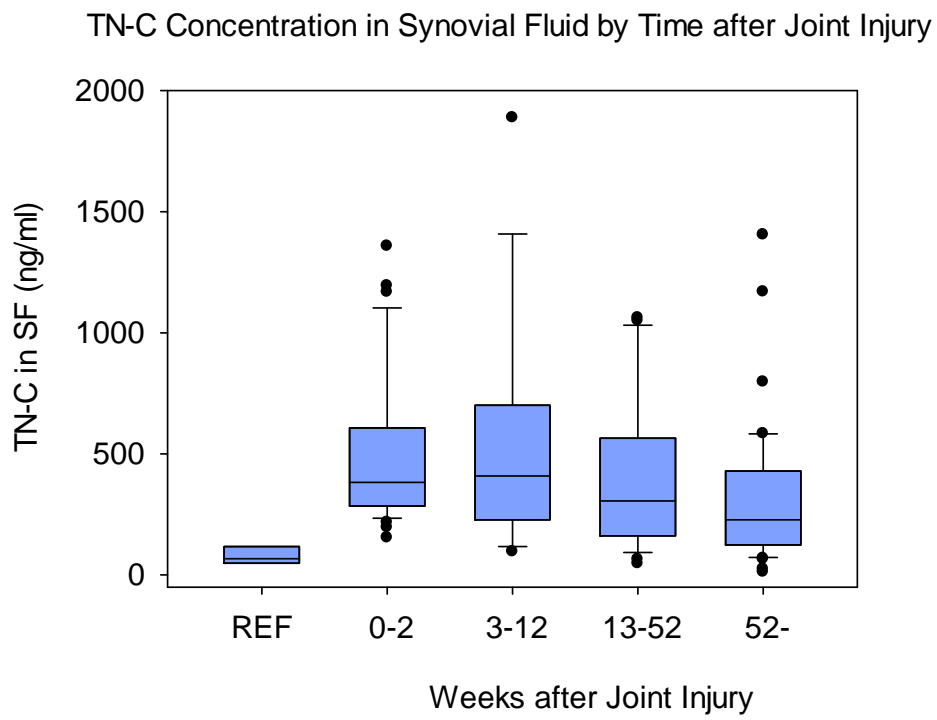
Figure 1

TN-C Concentration in Synovial Fluid by Diagnosis

Figure 2

TN-C Concentration in Synovial Fluid by Time after Joint Injury

Figure 3