PERIOSTIN AND TGFBI IN BREAST CANCER PROGRESSION

by

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ABSTRACT

MICHELLE MARISSA PHELPS. Periostin and TGFBI in breast cancer progression. (Under the direction of DR. DIDIER DRÉAU)

Dynamic interactions between cancer cells, supportive stromal cells, the extracellular matrix (ECM), and the immune system are critical to breast cancer progression. Periostin is an ECM protein correlated to poor outcomes in breast cancer. Periostin is structurally similar to another ECM protein TGFBI that plays an opposite role in cancer. The first portion of this thesis focused of periostin in breast cancer progression. First, the periostin/TGFBI ratio was associated with increased breast tumor size and progression in mice and humans. *In vitro*, breast cancer cells secreted periostin, which led to expression and activation of the cytokine TGF-β in a positive regulatory loop. In a mouse mammary cancer model, treatment with the angiotensin receptor blocker losartan, an upstream inhibitor of TGF-β production, decreased the periostin/TGFBI ratio and led to decreased cancer progression. Further *in vitro*, periostin decreased VEGF secretion, increased TGF- β secretion, inhibited adhesion, and decreased nonspecific phagocytosis of macrophages. *In vivo*, periostin pre-treated RAW macrophages co-injected with 4T1 cancer cells led to decreased tumor size compared to un-treated macrophages plus cancer cells. Together, the experimental observations indicated that the periostin/TGFBI expression ratio, which can be altered by losartan is associated with breast cancer progression, and, that periostin may also have paradoxical effects on macrophages. The second of portion of this thesis highlights multiple aspects of the management of orthopedic patients.

DEDICATION

To my loving grandmother

Mary Eulalia Coleman.

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CHAPTER 1: INTRODUCTION

This dissertation presents the results of research conducted during the author's graduate studies at the University of North Carolina at Charlotte. The author, a M.D./Ph.D. candidate, has pursued concomitantly basic science studies and clinically-related research during her PhD studies to foster her academic growth and maintain ties in her specialty, respectively.

The overarching theme of this body of work is the advancement of care of the orthopaedic patient. Broadly defined, clinical care includes the entire engagement of a patient with the health care system. This includes a patient's experience in accessing and navigating the healthcare system, receiving medical treatment and follow-up, and establishing relationships with healthcare providers that put the patient's disease process in context. Advancement of patient care can be achieved in diverse areas, as demonstrated through the following chapters.

Chapters 2 and 3 focus on basic science research conducted under the direction of Dr. Didier Dréau. Chapter 2 discusses the importance of the ratio of extracellular matrix proteins periostin and TGFBI in the progression of breast cancer, while Chapter 3 presents the paradoxical effects of periostin on tumor-associated macrophages. The common vein between these two chapters is the investigation of periostin, a protein that was originally identified in the bone and named osteoblast-specific factor-2 (Osf-2) (1). Periostin was later renamed due to its expression in the periosteum, a fibrous connective

tissue layer surrounding the bones (2-4). Through effects on osteoblasts, periostin plays a role in osteogenesis and maintenance of the bone micro-architecture (5-7). Furthermore, periostin has been found to be upregulated in musculoskeletal diseases including fractures (8), skeletal muscle injury (9), ligament injury (10), osteoarthritis (11), fibrous dysplasia (4), rheumatoid arthritis (12), and muscular dystrophy (13). Interestingly, periostin is also upregulated in a wide variety of common epithelial cancers that frequently metastasize to the bone, including breast, lung, renal, and prostate cancer (14, 15). Given periostin's importance in both musculoskeletal development and disease, the further study of this protein in the cancer setting is intriguing.

Chapters 4-6 present clinically-related projects completed through collaboration with the departments of orthopaedic surgery at both Carolinas Medical Center and Baylor College of Medicine. First, Chapter 4 presents an article reviewing the workup and outcomes of soft tissue sarcomas, conducted under the direction of Dr. Jeffrey Kneisl (16). Diagnosis and management of patients with soft tissue sarcomas is an integral part of the practice of musculoskeletal oncology, and evidence-based guidelines and algorithms are helpful in providing excellent orthopaedic patient care.

The scope of orthopaedic oncology also includes management of patients with operative bone metastases. Approximately 350,000 people in the United States die with metastatic bone disease each year (17), and bone metastases can cause severe chronic pain, hypercalcemia, leukoerythroblastic anemia, pathologic fractures, and spinal cord compression (18). Chapter 5 presents the report of a patient with a bone metastastatic pheochromocytoma, an uncommon presentation encountered by the

orthopaedic surgeon. This chapter emphasizes important steps in clinical decision-making and management for these unique patients.

Lastly, Chapter 6 presents an original research article investigating factors affecting orthopaedic patient follow-up in clinic, conducted under the direction of Dr. Charles A. Reitman (19). In the provision of patient care, it is important not only to understand the biology of human disease, but also to understand factors that affect patients' utilization or access to care (20, 21). In this chapter, the patient-physician interaction and the orthopaedic management of patients in the emergency room are demonstrated to impact patient follow-up.

From understanding the biology of cancer, to managing patients in an evidence-based manner, to exploring the determinants of patient utilization of healthcare resources, advancements can be made which impact the care of orthoapedic patients.

CHAPTER 2: LOSARTAN ALTERS THE RATIO OF THE EXTRACELLULAR MATRIX PROTEINS PERIOSTIN AND TGFBI AND DECREASES BREAST CANCER PROGRESSION IN VIVO

2.1 Abstract

Periostin and transforming growth factor beta-induced (TGFBI) are extracellular matrix proteins with structural and functional similarities but opposite roles in breast cancer progression. The co-expression of these proteins within the tumor microenvironment is unknown. Expression of periostin and TGFBI was determined in human breast cancer specimens. An increased periostin/TGFBI expression ratio was associated with increased tumor size and advanced cancer stage. *In vitro*, breast cancer cells expressed both periostin and TGFBI, and more aggressive cancer cells tended to express an increased periostin/TGFBI ratio. Treatment with TGF-β increased periostin expression by cancer cells, and periostin, in turn, increased expression and activation of TGF-β in a positive regulatory loop. In a mouse mammary cancer model, treatment with the angiotensin receptor blocker losartan, an upstream inhibitor of TGF-β production, decreased the periostin/TGFBI ratio and led to decreased cancer progression. The periostin/TGFBI expression ratio in the primary tumor and plasma, regardless of treatment, was associated with increased bone metastasis. Taken together, the periostin/TGFBI expression ratio is associated with breast cancer progression and can be altered by losartan, a novel potential adjuvant therapy for breast cancer treatment.

2.2 Introduction

Breast cancer is the second leading cancer cause of death amongst U.S. women (22). Metastatic disease portends the poorest prognosis, and current treatment options for advanced-stage patients are limited (23). Periostin (also known as OSF2, PN, POSTN) is an extracellular matrix (ECM) protein that is upregulated in many cancers and is associated with poor prognosis in breast cancer (14, 24-29). Periosin plays a role in development and tissue repair but is generally not expressed in healthy adult tissues (8-10, 13, 30-32). *In vitro* and *in vivo* studies demonstrate that periostin promotes proliferation, survival, migration, invasion, and metastasis of breast cancer cells (14, 15, 25-27, 33, 34). Interestingly, periostin possesses similar protein structure and binding domains to another ECM protein, transforming growth factor beta-induced (TGFBI, also known as β-Ig-H3, (Fig. 1). The homologous N-terminal regions of POSTN and TGFBI contain four fascilin I domains that bind integrins and an EMI Domain which binds collagen-I and fibronectin. Only a small portion of the C-terminal regions of periostin and TGFBI differ.

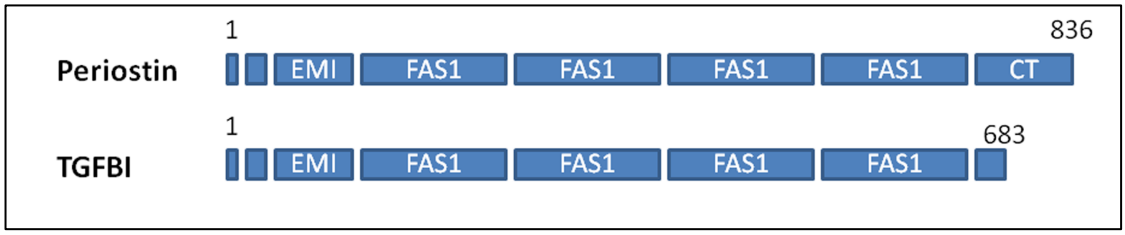


Figure 1: Periostin and TGFBI Protein Structure. Human periostin contains 836 amino acids, while TGFBI contains 683.

TGFBI is ubiquitously expressed in normal adult human tissues (35), but unlike periostin, TGFBI expression is down-regulated in a variety of human tumors (35-37). In fact, TGFBI appears to play an opposite role to periostin, leading to decreased cancer aggressiveness and metastasis in mouse models (38, 39). Both of these proteins are secreted by stromal cells in response to the pro-fibrotic cytokine TGF-β (1, 35, 40). In addition, both periostin and TGFBI's effects are mediated through integrin signaling (41, 42), activating diverse intracellular signaling pathways (35, 43, 44). Previous studies have investigated either periostin or TGFBI expression in cancer tissues individually, and whether these proteins are co-expressed within the tumor microenvironment is unknown.

Angiotensin is a circulating hormone which plays important roles in vascular physiology and the endocrine system(45). Angiotensin promotes vasoconstriction of blood vessels and stimulates the release of aldosterone from the adrenal cortex, leading to an increase in blood pressure. For this reason, the angiotensin receptor blocker (ARB) losartan is used clinically for the treatment of hypertension (45). Through diverse downstream effects of angiotensin inhibition, ARBs have been shown to effectively decrease TGF-β and periostin expression in murine models of muscular dystrophy, myocardial infarction, and chronic kidney disease (46-48). While no prospective human trials have evaluated the use of ARBs for cancer treatment, a recent systematic review documented several studies which correlated ARB or other anti-RAAS medication use to improved outcomes in cancer patients (49). Furthermore, in a xenograft mouse mammary tumor study, ARB treatment led to decreased primary tumor

size (50). To our knowledge, no group has investigated the effect of ARBs on breast cancer metastases, the primary cause of mortality in breast cancer patients.

In this study, we first show that a high ratio of periostin/TGFBI expression in human primary breast cancer tumor correlates with worsened outcomes. Next, we demonstrate that *in vitro* the expression of periostin and TGFBI by breast cancer cells is in part regulated by a positive feedback loop between periostin and TGF-β signaling. Lastly, we demonstrate that treatment with losartan, an upstream inhibitor of TGF-β and periostin expression alters the periostin/TGFBI ratio and decreases tumor progression *in vivo*.

2.3 Materials and Methods

Reagents and antibodies

Human recombinant periostin protein was purchased from Sino Biological Inc. (Beijing, China). Human recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Losartan potassium was purchased from TCI America (Portland, OR). Angiotensin II was purchased from MP Biomedicals, LLC (Solon, OH). Anti-periostin antibody specific to amino acid residues 787-836 in the C-terminal region of the protein (ab83739) was purchased from Abcam Inc. (Cambridge, MA). Anti-TGFBI antibody specific to residues 626-683 in the C-terminal region (sc-28660) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Anti-TGF-β1 (sc-146), anti-TGF-β2 (sc-90), and anti-TGF-β3 (sc-82) were also purchased from Santa Cruz Biotechnology Inc.

Breast cancer patient tissue arrays

Human breast tissue arrays with serial sections of 61 tumor specimens were obtained from BioChain (Newark, CA). Clinical information (gender, age, tumor size, presence of positive lymph nodes, presence of metastases, hormone receptor positivity, and histologic subtype) was provided for each specimen included. Tissue arrays were stained with hematoxylin and eosin and used in immunohistochemistry analyses of periostin and TGFBI protein expression.

Cells and culture conditions

Human breast epithelial cells (MCF10A), human breast carcinoma cells (MCF7 and MDA-MB-231 (abbreviated as 231 hereto forth)), and mouse mammary carcinoma cells (4T1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Red fluorescent protein (RFP)-expressing 4T1 cancer cells were obtained from Anticancer Inc. (San Diego, CA). Media and supplements were obtained from Hyclone (Logan, UT) unless noted. 4T1 cells were cultured at 37°C and 5% CO_2 in DMEM media supplemented with 10% fetal bovine serum (FBS; Atlanta Biologics, Atlanta, GA), gentamycin, and amphotericin B. MCF7 and MDA-MB-231 were cultured in DMEM/F12 media supplemented with 10% FBS and antibiotics as above. MCF10A cells were cultured in DMEM/F12 media with 5% horse serum, 10 μg/ml insulin (Sigma-Aldrich, St Louis, MO), 20 ng/ml epidermal growth factor (eBioscience, San Diego, CA), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), and antibiotics. For all *in vitro* experiments, cells (3-5 x 10^5 per well) were seeded in 6-well plates and starved in 0% FBS media for 24 hours prior to treatment for 24 to 48 hours.

Orthotopic murine mammary cancer model

Female Balb/C mice (Jackson Lab, Bar Harbor, ME) were housed and maintained in the Vivarium in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of North Carolina at Charlotte. Mice were injected subcutaneously in an inguinal mammary fat pad with 3 x 10^5 RFP-expressing 4T1 cancer cells in 100 µl of phosphate buffered saline (PBS). Mice were randomly assigned to treatment with control versus losartan *po* in drinking water at a dose of 10-15 mg/kg/day. Tumor growth was assessed by fluorescence (i.e., radiant efficiency ([p/s/cm²/sr]/[µW/cm²]) determined over a 30-day period using an *in vivo* imaging system and the Living Image software package (IVIS, Perkin Elmer, Waltham, MA). Mice were euthanized at day 30, and blood and organs were harvested including the lungs, liver, spine, femurs, tibias, humeri, and spleen. Distant metastasis was assessed by measuring the fluorescence emitted by tumor cells in these harvested organs using the IVIS system. Primary tumors were assessed for protein expression of periostin and TGFBI by immunohistochemistry (see below). Following collection in heparinized tubes, plasma was isolated by centrifugation (51). Plasma levels of periostin and TGFBI protein were assessed by enzyme-linked immunosorbent assay (ELISA, see below). The periostin/TGFBI ratio was determined by dividing the two protein levels. A high ratio was defined as any ratio above the average for all samples combined, while a low ratio was defined as any ratio below the average.

Western blot

Cells were washed in PBS then lysed with PROPREP™ protein extraction solution (iNtRON Biotechnology, Kyungki-Do, Korea). Western blot analysis of

protein expression was performed as previously described (52). Briefly, samples containing equal protein amounts were denatured, loaded onto 10% polyacrylamide gels, and separated with SDS-PAGE electrophoresis. Proteins were then transferred to a nitrocellulose membrane using a semi-dry transblot apparatus (Biorad, Hercules, CA). The quality of the transfer was assessed through reversible Ponceau S staining (0.1%, Sigma). Membranes were blocked with Tris-buffered saline - 0.1% Tween 20 containing 5% nonfat milk and then incubated with appropriate primary antibodies. Following washing, membranes were incubated with a species-specific horseradish peroxidase (HRP)-conjugated secondary IgG antibody (Jackson ImmunoResearch, West Grove, PA). After additional washing, membranes were incubated with chemiluminescent HRP substrate (Thermo Fisher Scientific Inc., Waltham, MA) and signal was detected using the UVP imaging system and the VisionWork software (UVP, Upland, CA). Intensities of protein bands were semi-quantified using QuantityOne software (Biorad, Hercules, CA). Protein expression was normalized to expression of a loading control (β-actin).

Enzyme-linked immunosorbent assays (ELISA)

Periostin, TGFBI, and TGF-β1 concentrations in human and murine cell culture supernatants were assessed using ELISA kits (R&D Systems, Minneapolis, IN) according to the manufacturer‘s recommendations. The absorbance of each sample along with that of a standard curve was determined using a microplate reader (Biotek, Winooski, VT) and the concentrations (pg/mL) of proteins were derived from the standard curve. Periostin and TGFBI levels in mouse plasma were also determined.

For these determinations, standards were diluted in in PBS containing 20% FBS, and plasma samples were diluted 1:60 in PBS containing 20% FBS.

Histology, immunofluorescence staining (IF), and immunohistochemistry (IHC)

Both human arrays and mouse samples were stained with hematoxylin and eosin to identify the tumor mass. The presence of periostin or TGFBI in human breast tumors was assessed using human tissue arrays and immunofluorescence as described earlier (53). Briefly, after immunostaining with a specific primary antibody, specimens were incubated with a secondary antibody conjugated to fluorophore Alexa 633 (BD Biosciences, San Jose, CA). DAPI was used as a nuclear counter stain (Life Technology, Grand Island, NY). For each protein, expression (IF intensity) was quantified using using CellProfiler software (54) and normalized to the number of nuclei present. Background staining from a negative IgG control was subtracted. The Periostin/TGFBI ratio was calculated by dividing the IF intensities.

For murine tumor samples, immunohistochemistry was performed on serial sections (5-6 μm) of paraffin-embedded primary mammary tumors to assess for the presence of periostin and TGFBI, as detailed previously (51). Briefly, after immunostaining with a specific primary antibody, specimens were incubated with a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Detection of protein expression was carried out using the Vectastain Universal horseradish peroxidase system (Vector Laboratory, Burlingame, CA) according to the manufacturer's protocol. All stains included a negative IgG control to assess background staining. Protein expression was quantified using a grading scale with two components: an intensity score (0-100%) and a distribution score (0-100%

coverage of tumor area). Background staining of the control IgG was taken into account during scoring. Tumor ratio of periostin/TGFBI was defined as a high ratio if the visual score for periostin was higher than the score for TGFBI expression. A low ratio was defined as a tumor having a higher TGFBI visual score than the score for periostin expression.

Statistical analysis

All data are presented as means ± standard error of the mean (SEM) unless otherwise noted. Statistical significance was determined using Prism software (Graphpad Software, Inc., La Jolla, CA). Experiments were analyzed using t-tests (two groups) or one-way ANOVA followed by Tukey's post-hoc test (3 or more groups, one factor). Experiments with two factors were analyzed with two-way ANOVA, followed by post-hoc tests. Repeated measures were used as indicated for *in vivo* study analysis. Pearson's correlation was used to investigate the relationship between continuous variables. Data with a non-normal distribution was normalized using a log transformation as indicated. Significance was set *a priori* to p value below 0.05, two-tailed.

2.4 Results

Periostin/TGFBI expression ratio correlates with human breast cancer progression

Given previous reports of the opposite effects of periostin and TGFBI in human breast (33, 34, 38, 39), we first investigated whether periostin and TGFBI are co-expressed within the tumor microenvironment. Expression of periostin and TGFBI protein expression was determined a cohort of 61 primary human breast cancer samples. Median age of the patients was 50 years old, and most of the patients included were

classified as American Joint Committee on Cancer (AJCC) stage II-IV (Table 1). Antibodies specific to the non-homologous C-terminal regions of both periostin and TGFBI were utilized to assess expression in primary tumors. While the expression of these proteins was highly variable, the periostin/TGFBI expression ratio correlated with clinical parameters (Fig. 2). An increased periostin/TGFBI ratio significantly correlated with tumor size, although this was a weak relationship (Fig. 2A). Also, an increased periostin/TGFBI ratio was associated with increasing AJCC stage (Fig. 2B). This finding remained significant when patients were grouped into Stage I-II vs. Stage III-IV.

Periostin/TGFBI expression ratio varies between human breast cells

There are conflicting accounts in the literature regarding whether cancer cells are able to express periostin, as periostin expression is generally attributed to stromal cells (25, 26, 29, 40, 55, 56). Therefore, we next investigated periostin expression in a human breast progression series *in vitro.* MCF10A, MCF7, and 231 cells all expressed periostin and TGFBI proteins as assessed by western blotting of cellular lysate (Fig. 3A). Periostin expression was the highest in MCF7 cells compared to MCF10A and 231 (Fig. 3B). The three cell lines expressed TGFBI as demonstrated by bands at three expected molecular weights, however the banding pattern was different between the cell lines (Fig. 3A). For quantification, densitometry was performed for the three individual bands and summed together to equal total TGFBI protein expression. MCF7 cells expressed the highest total level of TGFBI compared to MF10A and 231 (Fig. 3C). Next, we investigated the periostin/TGFBI expression ratio in these cells. The ratio of

periostin/TGFBI protein expression tended to increase with increasing aggressiveness, in concordance with our observations in human breast tissues (Fig 3D).

Table 1: Clinical characteristics of patients included in the human breast tissue array.	
Female (%)	100%
Mean Age in Years (Median)	51 (50)
Estrogen Receptor Positive	68%
Progesterone Receptor Positive	58%
Invasive Ductal Carcinoma Other	87% 13%
Mean Volume in cm^3 (Median)	56 (23)
AJCC Breast Cancer Stage	Stage 1 – 2% Stage 2 – 50% Stage 3 – 23% Stage 4 – 25%

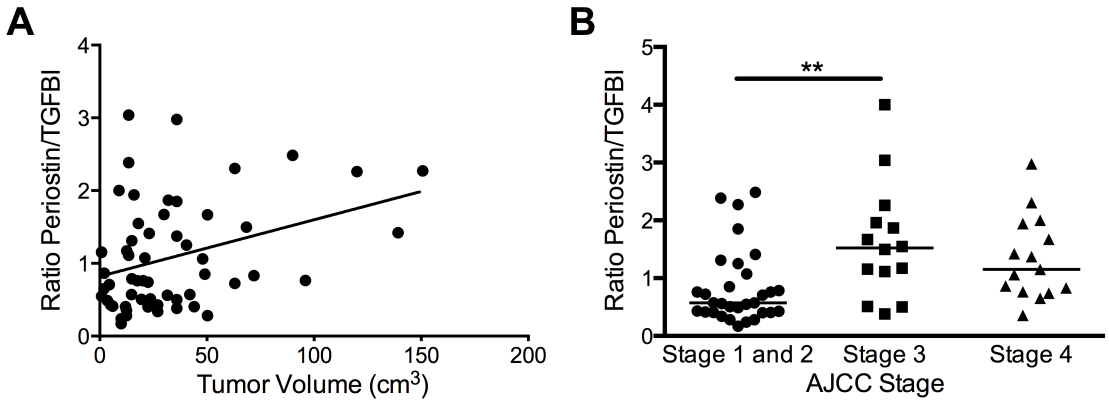


Figure 2: The expression ratio of periostin/TGFBI correlates with human breast cancer progression. Serial sections were immunostained for periostin and TGFBI protein expression. Following normalization to the number of nuclei present, the ratio of periostin/TGFBI expression was determined. (A) The periostin/TGFBI ratio correlates with increasing tumor volume ($p=0.007$, $R^2=0.12$). (B) The periostin/TGFBI ratio is associated with increased AJCC Stage (ANOVA $p=0.004$, $^{**}p<0.01$).

In cancer cells, TGF-β and periostin participate in an autocrine positive feedback loop unaffected by angiotensin and losartan treatments in vitro

We next evaluated which signals within the tumor microenvironment modulate periostin expression by cancer cells. TGF-β1 has previously been shown to increase secretion of both periostin and TGFBI by fibroblasts and other mesenchymal cells (1, 40, 57). Indeed, exogenous TGF-β1 treatment of both MCF7 cells (Fig. 4AB) and 4T1 cells (Fig 4C) led to a significant increase in periostin expression.

As periostin knockout mice have decreased TGF-β1 expression in their tissues (13, 58), we hypothesized that periostin participates in a positive feedback regulatory loop with TGF-β. Indeed, exogenous periostin treatment significantly increased expression of TGF-β1 by 4T1 cancer cells at 24 hours in a dose-dependent manner (Fig. 5A and B). Furthermore, periostin treatment significantly increased expression of TGF-β3, but not TGF-β2 (Fig. 5A and B). Moreover, periostin treatment for 48 hours led to increased activation of TGF-β1, as assessed by ELISA of 4T1 culture supernatant (Fig. 5C).

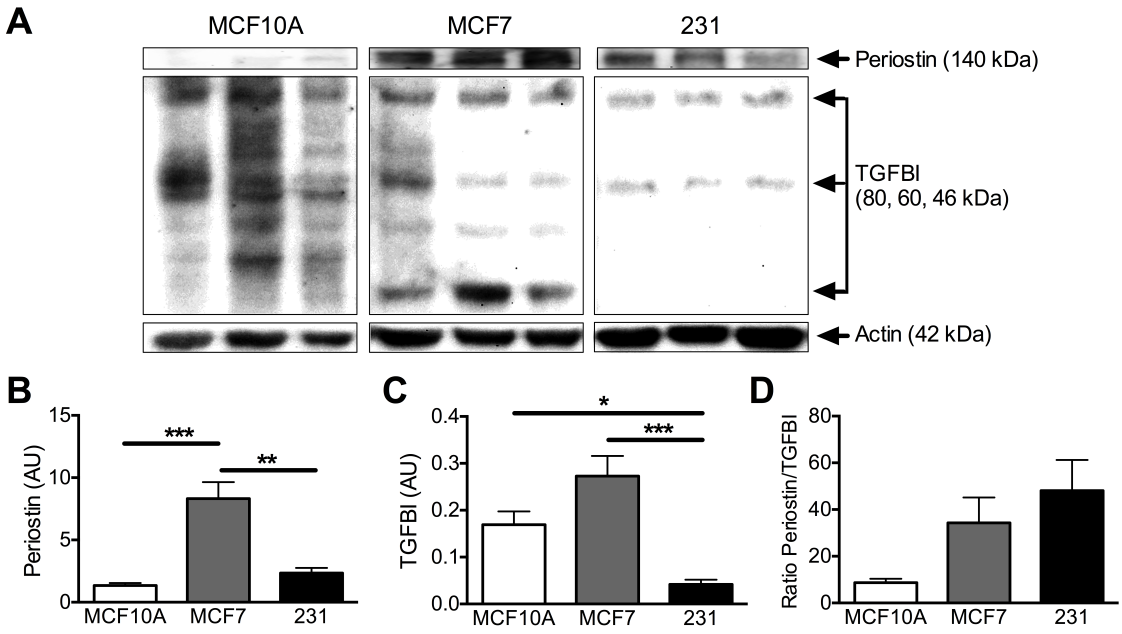


Figure 3: The expression ratio of periostin/TGFBI is variable in a human breast cell series. (A) Intracellular expression of periostin and TGFBI was evaluated in three human breast cells by western blot (MCF10A, MCF7, and MDA-MB-231 (abbreviated to 231)). (B) Quantification of periostin expression (140 kDa, ANOVA p=0.0003). (C) Quantification of total TGFBI expression (combined expression of bands at 80, 60, and 46 kDa, ANOVA p=0.0005). (D) Ratio of periostin/TGFBI by cell line (ANOVA p=0.0539). *p<0.05, **p<0.01, ***p<0.001.

Additionally, we investigated the angiotensin II-AT1R pathway upstream of TGF-β that may affect periostin or TGFBI secretion by cancer cells. Angiotensin is known to increase secretion of TGF-β and ECM proteins by stromal cells in breast cancer and other diseases (57, 59, 60). Interestingly, angiotensin receptor blockers abrogate the effect of losartan, decreasing TGF-β and periostin expression in mouse models of muscular dystrophy, myocardial infarction and chronic kidney disease (46-48). Therefore, we next determined the effects of angiotensin and losartan on breast cancer cell secretion of periostin and TGFBI. Treatment of 4T1 cancer cells with increasing concentrations of angiotensin II (0.1-10uM) for 24-48 hours did not affect

TGF-β, periostin, or TGFBI secretion (data not shown). Furthermore, losartan alone (0.1-100 μM), or in combination with angiotensin II did not affect 4T1 secretion of periostin or TGFBI (data not shown).

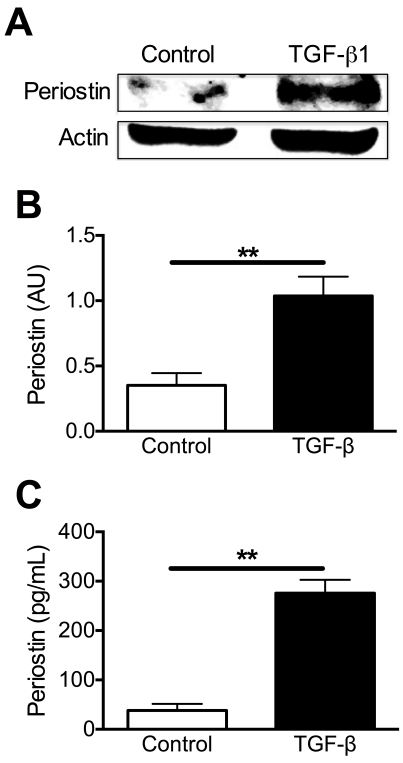


Figure 4: TGF-β1 treatment increases periostin expression by breast cancer cells. After treatment for 24 hours with TGF-β1 (20 ng/mL), expression of periostin by MCF7 human breast cancer cells was evaluated by western blotting of cellular lysate. (A) Representative western blots. (B) Quantification of periostin expression (student's t-test **$p<0.01$). (C) 4T1 murine mammary cancer cells were treated for 48 hours with TGF-β1 (20 ng/mL). ELISA was performed of the culture supernatant to assess periostin secretion (student t-test **$p<0.01$).

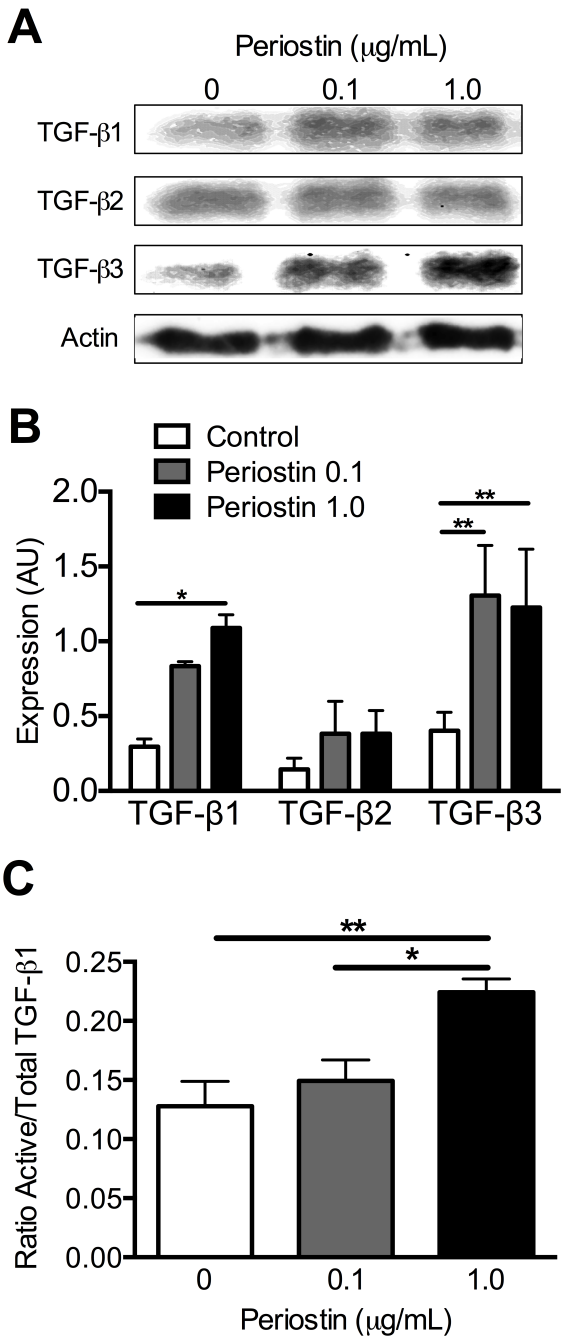


Figure 5: Periostin treatment increases TGF-β expression and activation. (A) 4T1 cells were treated for 24 hours with a dose curve of periostin (0, 0.1, and 1.0 μg/mL). Western blotting was performed on cellular lysate, and representative bands at 12.5 kDa are shown. (B) Quantification of expression of TGF-β1, TGF-β2, and TGF-β3 (two-way ANOVA, periostin treatment p=0.0009, TGF-β type p=0.0013). (C) Activation of TGF-β1 in culture supernatant was assessed after treating 4T1 cells for 48 hours with a periostin dose curve (ANOVA p=0.0077). *p<0.05, **p<0.01.

Losartan treatment decreases the periostin/TGFBI ratio and inhibits mammary tumor progression *in vivo*

Given the importance of the periostin/TGFBI ratio suggested by our investigation of human breast tissues, we next aimed to investigate a treatment that might alter this ratio, leading to improved outcomes *in vivo*. Our *in vitro* studies indicated the key role of TGF-β in regulating periostin and TGFBI expression. Although angiotensin and losartan did not directly affect cancer cell secretion of these ECM proteins *in vitro*, others have found that losartan decreases TGF-β and expression by cancer-associated fibroblasts isolated from breast cancer biopsies (61). Therefore, we hypothesized that losartan treatment would alter the periostin/TGFBI ratio, potentially through an effect on stromal cells, leading to decreased mammary tumor progression. To test this, 4T1-RFP cancer cells were injected subcutaneously into the mammary fat pad of 19 female Balb/C mice. Mice were dosed orally for 30 days with control drinking water or with losartan-infused water (10-15 mg/kg/day).

Losartan treatment led to significantly decreased primary tumor size compared to those observed in control mice, as assessed by fluorescence (Fig. 6A and B). Importantly, losartan treatment led to significantly decreased distant metastasis, preventing the formation of bone metastases (Figure 6C and D).

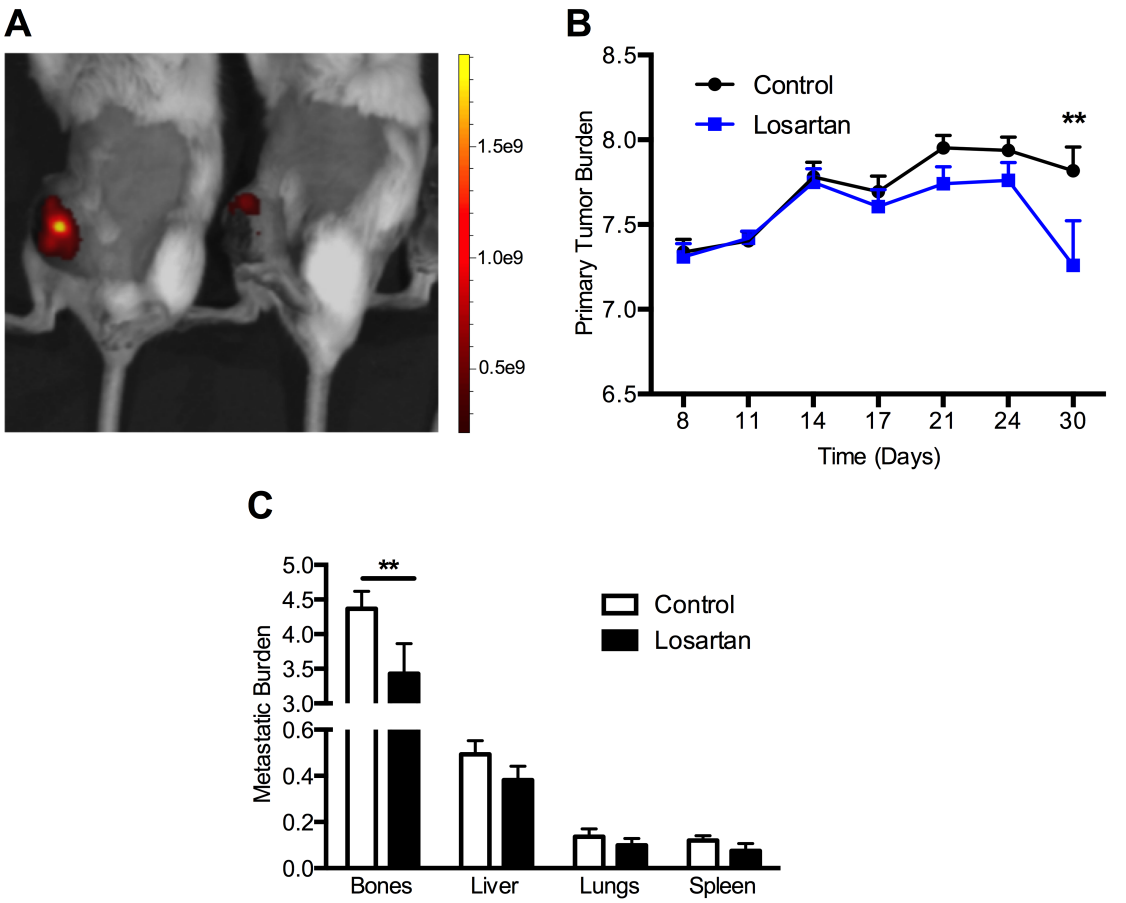


Figure 6: Losartan treatment decreases mammary tumor size and bone metastasis. 4T1-RFP cells were subcutaneously injected in female Balb/C mice. (A) Primary tumor size was assessed by fluorescence intensity over time for 30 days. (B) Losartan treatment decreased primary tumor size by day 30 (primary tumor burden = log [fluorescent radiant efficiency], two-way repeated measures ANOVA, time $p<0.0001$, treatment $p=0.15$, interaction $p=0.047$, day 30 control versus losartan $p<0.01$). (C) Losartan treatment decreased distant metastasis to the bones (metastatic burden = fluorescent radiant efficiency x 10^{-7}, two-way ANOVA, metastatic site $p<0.0001$, treatment $p=0.035$, interaction $p=0.049$, bone metastasis control vs. losartan $p<0.01$). (D) The bone metastatic burden (fluorescent radiant efficiency x 10^{-7}) was compared between the lower extremity (femur and tibia), upper extremity (humerus), and spine. Losartan significantly decreased lower extremity bone metastases (two-way ANOVA, metastatic site $p<0.0001$, losartan treatment $p=0.0079$, lower extremity control vs. losartan $p<0.05$).

Immunohistochemistry of primary mammary tumors revealed a trend towards losartan decreasing periostin expression ($p=0.065$, Fig. 7A and B). Meanwhile, losartan significantly increased TGFBI expression in primary tumors (Fig. 7C and D).

Interestingly, most of the mice expressed periostin or TGFBI, but did not co-express these proteins to the degree seen with human tumors. The absence of expression of either periostin or TGFBI in many primary tumor samples prevented the derivation of the expression periostin to TGFBI ratio.

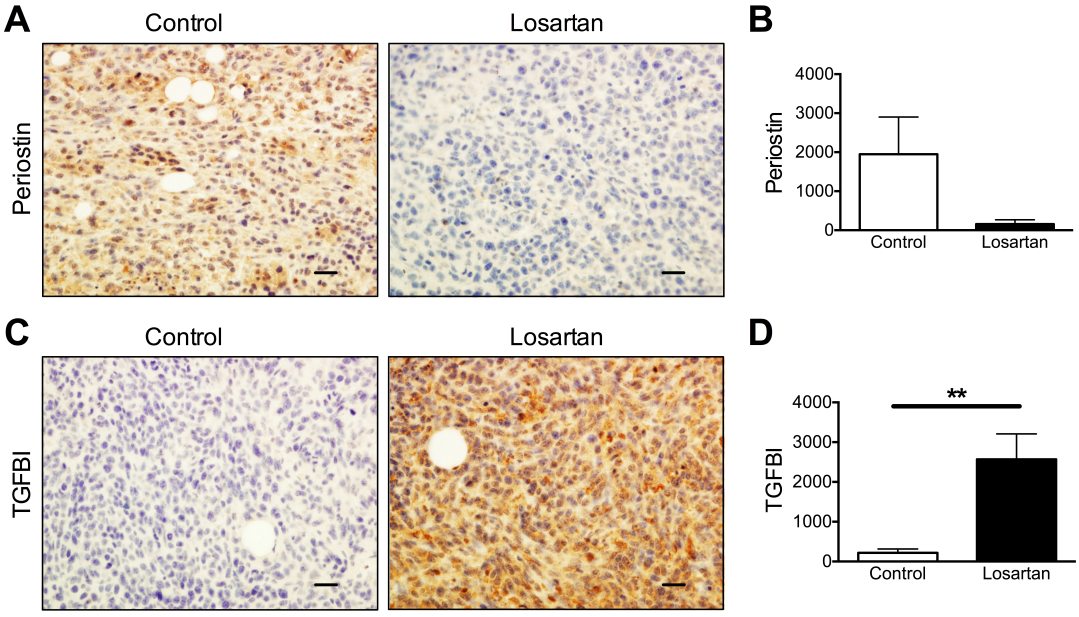


Figure 7: Losartan treatment alters periostin and TGFBI expression in the primary tumor. Expression of periostin and TGFBI in murine mammary tumors was assessed by IHC. (A) Representative microphotographs of periostin staining in control versus losartan-treated mice (scale bar=100 μm). (B) Losartan tended to decrease periostin expression in the primary tumor (student's t-test p=0.065). (C) Representative microphotographs of TGFBI expression in control versus losartan-treated mice. (D) Losartan treatment significantly increased TGFBI expression in the primary tumor (student's t-test p=0.0031).

Remarkably, losartan did alter the ratio of periostin/TGFBI in murine plasma (Fig. 8). Periostin plasma levels were higher than expected and did not different between control and losartan-treated mice (Fig. 8A). However, losartan significantly increased TGFBI plasma levels (Fig. 8B), leading to an overall decrease in the ratio of periostin/TGFBI in losartan-treated mice (Fig. 8C).

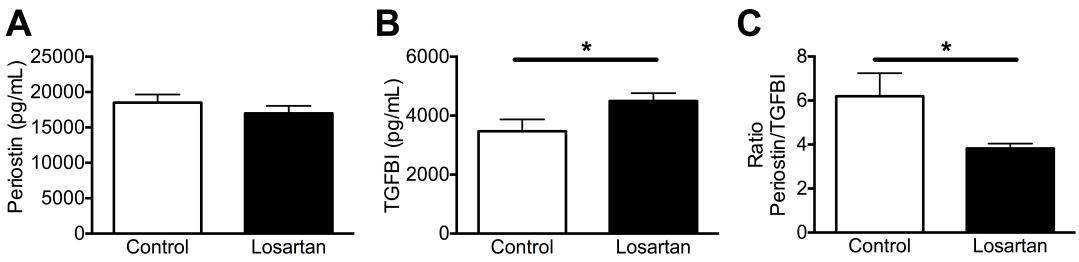


Figure 8: Losartan alters the periostin/TGFBI ratio in murine plasma. Plasma was collected at euthanasia on day 30. ELISAs were performed to assess protein levels (A and B), and the ratio was evaluated (C). (A) The level of periostin protein in mouse plasma was unaffected by losartan treatment (student's t-test, n.s.). (B) Mouse plasma levels of TGFBI were significantly increased by losartan treatment (student's t-test p=0.0496). (D) The ratio of periostin/TGFBI expression was significantly decreased by losartan treatment (student's t-test p=0.0421).

Periostin/TGFBI expression ratio correlates with distant bone metastasis *in vivo*

To further investigate the prognostic potential of the periostin/TGFBI expression ratio, primary tumors and plasma from mice were next classified as having a high ratio (more periostin) or a low ratio (more TGFBI), regardless of treatment (Fig. 9). Mice with a high primary tumor periostin/TGFBI expression ratio had significantly more bone metastases (Fig. 9A). Classifying mice according to their plasma periostin/TGFBI ratio generated strikingly similar results. A high periostin/TGFBI expression ratio, regardless of treatment, was again associated with significantly increased metastases to the bones (Fig. 9B).

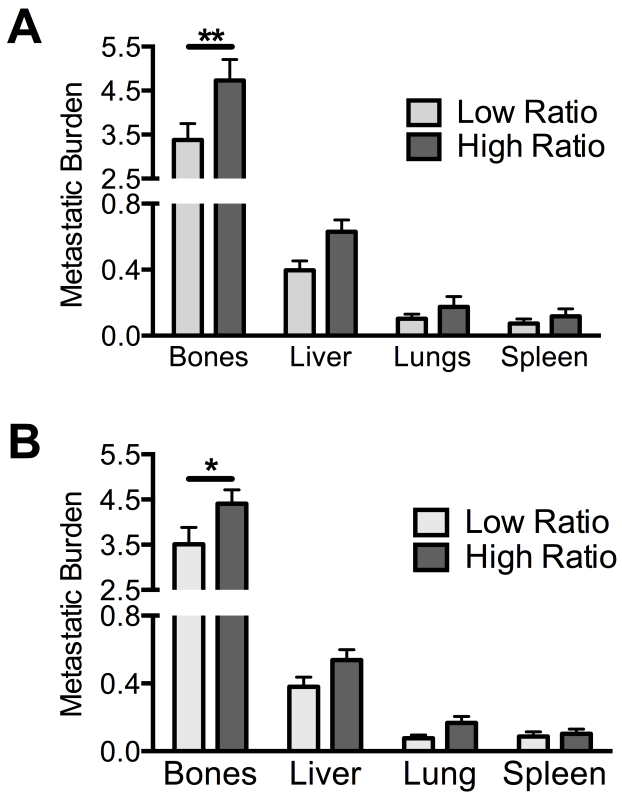


Figure 9: The ratio of periostin/TGFBI expression in primary tumors and plasma correlates with distant bone metastasis in vivo. The ratio of periostin/TGFBI protein expression in primary tumors (A) and plasma (B) was categorized as high vs. low, regardless of losartan treatment. The metastatic burden (fluorescenct radiant efficiency x 10^7) in distant organs (bones, liver, lungs and spleen) was compared between mice with high and low ratios. (A) A high periostin/TGFBI ratio in the primary tumor correlated with increased distant metastases to the bones (two-way ANOVA, metastatic site $p<0.0001$, ratio $p=0.019$, interaction $p=0.032$, bones low ratio vs. high ratio $p<0.01$). (B) A high periostin/TGFBI ratio in the plasma also correlated with increased metastases to the bones (two-way repeated measures ANOVA, metastatic site $p<0.0001$, ratio n.s., bones low ratio vs. high ratio $p<0.05$).

2.5 Discussion

The goals of this study were to investigate the expression of periostin and TGFBI in breast cancer, to determine the importance of the ratio of these proteins, and to alter the periostin/TGFBI ratio *in vivo* to affect outcomes using the ARB losartan. First, our data indicate that both periostin and TGFBI are co-expressed in both human and murine breast tumors, and that the periostin/TGFBI expression ratio correlates with breast cancer progression. Importantly, in this study we utilized antibodies raised against epitopes in the C-terminal regions of periostin and TGFBI, preventing non-specific cross-recognition of these structurally similar proteins. Our data also demonstrates the importance of the periostin/TGFBI ratio in breast cancer, a phenomenon reported here for the first time.

Our IHC analysis of human breast cancer specimens demonstrated that the periostin/TGFBI ratio correlates with increased tumor size and AJCC stage, and our *in vivo* investigation using the 4T1 orthotopic breast cancer model confirmed that a higher ratio of periostin/TGFBI was associated with increased distant metastasis to bones. However, the importance of the periostin/TGFBI ratio as a prognostic indicator versus a mechanistically causative signaling cooperation warrants further investigation.

Further, our data indicate that breast cancer cells express both periostin and TGFBI. While previous studies have presented conflicting results regarding the ability of cancer cells to express periostin (24-26, 28, 29, 62-64), our data in multiple murine mammary and human breast cancer cells strongly suggest the expression and secretion of periostin *in vitro*. This evidence supports the modulation by cancer cells of periostin concentrations within the tumor microenvironment.

Additionally, our results indicate that TGF-β1 increased expression of periostin, and that subsequent treatment with exogenous periostin increased expression of both TGF-β1 and TGF-β3 by breast cancer cells. Previous studies demonstrated that TGF-β affects secretion of periostin and TGFBI by stromal cells (40, 65) human mammary epithelial cells (66), and one human breast cancer cell line (67). Our observations of the effects of TGF-β and periostin on expressions by 4T1 cancer cells of periostin and TGF-β, respectively support a periostin – TGF-β autocrine regulatory loop in breast cancer comparable to those demonstrated in pancreatic cancer and bronchial epithelial cells (68, 69). In the wound healing setting, a positive regulatory loop between periostin and TGF- β could potentially promote angiogenesis, activation of fibroblasts, and ECM remodeling. During the inflammatory and proliferative phases of wound healing, infiltrating immune cells and activated local stromal cells secrete pro-inflammatory cytokines and growth factors, many of which are known to increase periostin expression (citation). For example, TGF- β, platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), and fibroblast growth factor (FGF) in the wound may all contribute to periostin upregulation (70-72). Periostin may then further increase TGF- β expression, driving ECM protein expression, myofibroblast activation, and resolution of the acute inflammatory phase (72). The resolution of local inflammation by macrophages and neutrophils might decrease local cytokine and growth factor concentrations, leading to an overall decrease in periostin secretion, and ultimately dampening the periostin-TGF-β feedback loop as wound healing progresses(70, 71). Consistent with this, experimental observations suggest that periostin upregulation is transient in the wound healing response, resolving within 7-21 days, coinciding with the timing of resolution of

leukocyte accumulation (71, 73). While this regulatory loop may be dampened in the wound healing response through local changes in inflammation, in the cancer microenvironment, the chronic inflammatory milieu may dysregulate and promote continuation of this feedback loop, leading to the continued promotion of cancer cell growth and invasion. The contribution and importance of this feedback loop could be further investigated in vivo utilizing neutralizing antibodies or genetic ablation of TGF-β or periostin, examining the effect on expression on its feedback partner and the effect on cancer progression.

Next, our data demonstrate that losartan effectively alters the periostin/TGFBI ratio and inhibits mammary tumor progression *in vivo*. Losartan is an ARB used clinically for the treatment of hypertension (45). Interestingly, ARBs have been shown to effectively decrease TGF-β and periostin expression in murine models of muscular dystrophy, myocardial infarction, and chronic kidney disease (46-48). Losartan is an inhibitor of the angiotensin II type 1 receptor (AT_1R), which mediates the effects of the hormone angiotensin II. Angiotensin signaling via the AT_1R leads to pleoitrophic downstream signaling effects through both G-protein and non-G-protein-related pathways (74). Angiotensin in known to upregulate TGF-β expression by vascular smooth muscle cells, hepatic stellate cells, fibroblasts, renal epithelial cells, and myocardial cells (75-77). In cardiac myocytes, angiotensin-upregualtes TGF-β via NADPH oxidase, leading to activation of protein kinase C (PKC), p38 MAP kinase, and nuclear activating protein-1 (AP-1) (77, 78). The transcription factor complex c-fos/AP-1 has also been implicated in Angiotensin II-mediated TGF- β upregulation in smooth muscle cells and cardiac fibroblasts (79, 80). Angiotensin II is also able to

directly upregulate expression of several ECM proteins such as collagen and fibronectin through multiple pathways, and angiotensin activates a known transcription factor for periostin, c-Fos (70, 75). Interestingly, our *in vitro* studies did not demonstrate an effect of losartan or angiotensin II on cancer cell secretion of periostin and TGFBI. Therefore losartan's effect in altering the periostin/TGFBI ratio *in vivo* may be due to effects on stromal cells, which would be consistent with the previous observation that losartan decreases angiotensin-induced secretion of periostin by human fibroblasts (57).

Importantly, we found that losartan decreased primary tumor size, a finding consistent with a prior study utilizing an immunocompromised xenograft mouse model and a non-metastatic human breast cancer cell line (50). To our knowledge, this is the first evidence of an ARB decreasing breast cancer growth in an orthotopic immunocompetent model. Furthermore, this is the first evidence of an ARB reducing breast cancer metastasis *in vivo*, an essential contributor to breast cancer patient morbidity and mortality. In particular, losartan decreased bone metastasis, the most common cause of breast cancer metastasis affecting approximately 70-80% of women with advanced breast cancer (18). Bone metastases can cause severe chronic pain, hypercalcemia, leukoerythroblastic anemia, pathologic fractures, and spinal cord compression (18), and, therefore, medications which may prevent bone metastasis have the potential for broad impact on quality of life. Losartan is known to alter multiple pathways relevant to cancer progression (60), and therefore, these effects are not specifically limited to the effects mediated by any changes in TGF- β, periostin, and TGFBI expression.

In conclusion, this study is the first to demonstrate the importance of the periostin/TGFBI ratio in breast cancer and to utilize a novel approach for prevention of mammary cancer metastases *in vivo* using losartan, a currently approved and generically available anti-hypertensive medication with a known safety profile. This strategy has demonstrated promise in preventing mammary tumor progression and warrants additional studies investigating losartan's use as an adjuvant cancer therapy.

CHAPTER 3: PERIOSTIN PROMOTES BREAST CANCER THROUGH EFFECTS ON TUMOR-ASSOCIATED MACROPHAGES

3.1 Abstract

Interactions between cancer cells and immune cells are critical to breast cancer progression. We have previously shown that breast cancer cells secrete periostin, an extracellular matrix protein implicated in tumor progression. Here we evaluated 1) the effect of macrophage secretions on 4T1 breast cancer cell expression of periostin, 2) the effect of periostin on macrophage functions *in vitro*, and 3) the effect of periostin on tumor-associated macrophages *in vivo*. *In vitro*, following a 48-hour incubation with conditioned media (CM) obtained from J774 monocyte cells, 4T1 mammary cancer cells secreted significantly higher periostin concentrations as determined by ELISA. Periostin significantly decreased macrophage adhesion to fibronectin-coated plates and inhibited non-specific phagocytosis of polymer beads by murine J774 and RAW monocyte cells and primary bone marrow macrophages. Furthermore, incubation with periostin led to decreased VEGF secretion and increased TGF-β secretion by RAW Cells. Finally, in an immunocompetent orthotopic murine mammary cancer model, periostin pre-treated RAW macrophages co-injected with 4T1 cancer cells led to decreased tumor size compared to un-treated macrophages plus cancer cells. These observations indicate that the secretion of periostin by 4T1 cells is, in part, stimulated through paracrine communication with macrophages. Periostin then modulates essential

functions of tumor-associated macrophages including altering cytokine secretions, decreasing macrophage adhesion, and inhibiting phagocytosis and destruction of the ECM. Together, these specific effects lead to macrophage-mediated breast tumor suppression *in* vivo. Further understanding of periostin's effects on immune cells is needed prior to exploring anti-periostin strategies against breast cancer.

3.2 Introduction

Interactions between cancer cells and macrophages are critical to the development of breast cancer metastases (81). Macrophages may polarize along a spectrum of phenotypes, which are generally classified into M1 versus M2 macrophages based on function and secretions (Fig. 10)(82-87).

M1 macrophages are "classically activated" and function as pro-inflammatory "soldiers" that defend the host from microbial infections and tumors (82-87). In wound healing, M1 macrophages are involved in the early acute inflammatory response, mediating tissue damage and extracellular matrix (ECM) phagocytosis. M1 macrophages produce high levels of inflammatory cytokines (such as IL-1 and TNF-α) and they express high levels of inducible nitric oxide synthase (iNOS), which leads to production of nitric oxide (NO). M1 macrophages exhibit high cytotoxic activity against phagocytosed microorganisms and tumor cells.

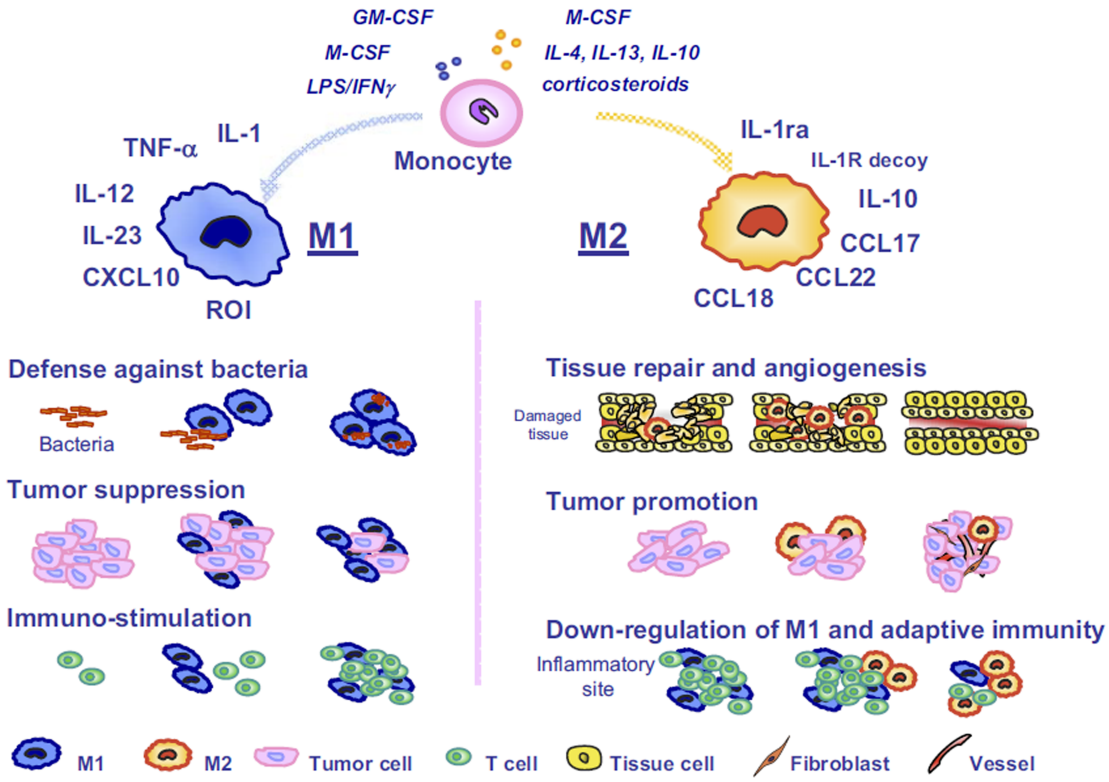


Figure 10: Polarization of macropahge function. From Solinas et al 2009.

M2 macrophages are "alternatively activated" and generally exhibit more diverse phenotypic spectra (82-87). For example, M2 macrophages may activate tissue remodeling and angiogenesis during wound healing. M2 macrophages can also dampen the inflammatory response and promote a Th2 response. In the tumor setting, M2 macrophages function in a pro-tumor capacity to help heal the "chronic wound" of the cancer microenvironment. M2 macrophages express high levels of arginase 1 and may produce an array of cytokines including IL-10 and TGF-β. M2 macrophages promote tumor initiation, progression, and metastasis via multiple mechanisms, including: pressing the angiogenic switch through secretion and activation of vascular endothelial growth factor (VEGF), promoting cancer cell proliferation through secretion of epidermal growth factor (EGF), and promoting matrix remodeling and invasion through

nonspecific phagocytosis and secretion of matrix metalloproteinases (MMPs) (81, 82). In vivo data suggest that M2-polarized macrophages directly promote breast cancer progression and metastatic spread (88, 89). A study of expression of CCL18-producing macrophages (M2-like) in 562 human breast cancer samples demonstrated that a high count of macrophages was associated with increased tumor size, stage, lymph node metastasis, distant metastasis, and decreased survival (90).

Macrophages that infiltrate a tumor are referred to as tumor-associated macrophages (TAMs). Importantly, while the M1 vs. M2 distinction is conceptually helpful, in vivo studies suggest that regardless of the pathology, macrophages are extremely plastic and within a single tumor assume a diverse range of phenotypes (91). The overall presence of TAMs has been found to correlate with worsened outcomes in many preclinical and human breast cancer studies (87, 88, 92), however, the specific phenotype of TAMs may affect overall prognosis (89, 93).

As discussed previously, periostin (also known as OSF2, PN, POSTN) is an extracellular matrix protein that plays a role in development and tissue repair (8-10, 13, 30-32). Periostin's effects are mediated through integrin signaling (41), which activates diverse signaling pathways, including NF-κB and others (43, 44). Periostin has been implicated in the progression of many types of cancer, and has been linked to poor patient outcomes in breast cancer (14, 24-29). In vitro and in vivo studies suggest that periostin promotes proliferation, survival, migration, invasion, epithelial to mesenchymal transition (EMT), and metastasis of breast cancer cells (14, 15, 25-27, 33, 34). Furthermore, periostin increases promotes activation of fibroblasts to cancer-

associated fibroblasts (94) and promotes angiogenesis through direct and indirect effects on endothelial cells (25).

Periostin plays a role in macrophage and other immune cell responses in several pathologic conditions that are mediated by chronic inflammation (13, 43, 95-97). For example, in allergic lung disease, periostin facilitates eosinophil tissue infiltration and adhesion to fibronectin (95). Similarly, in allergic skin disease, periostin knockout fibroblasts show decreased IL-4, IL-13, and IL-17a secretion compared to wild type fibroblasts (43). Periostin also plays a role in modulating macrophage function. In models of idiopathic pulmonary fibrosis and muscular dystrophy, periostin knockout mice show deficiencies in macrophage infiltration (13, 96). Furthermore, periostin promotes increased MMP-9 secretion from bone marrow-derived macrophages (97). Macrophages do express integrins, which play an important role in their phagocytosis (98-101), chemotaxis (99, 102), survival (102), and inflammatory responses (101, 103). Integrin signaling affects diverse macrophage signaling pathways. In particular, activation of NF-κB signaling in macrophages affects macrophage polarization and function in a context-specific manner (104).

Although periostin has clear roles in tumor promotion and has been linked to alteration of immune responses in several diseases, periostin's role in modulating macrophage behavior in the cancer microenvironment is unclear. The goals of the current study were to investigate 1) the effect of macrophages on expression of periostin in the breast cancer microenvironment, 2) the effects of periostin on macrophage functions *in vitro*, and 3) the effect of periostin on tumor-associated macrophages *in vivo*.

3.3 Materials and Methods

Reagents and antibodies

Human recombinant periostin protein was purchased from Sino Biological Inc. (Beijing, China). Human recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Lipopolysaccharide (LPS) was purchased from Sigma Aldrich (St. Louis, MO) Phorbol myristate acid (PMA) was purchased from Fisher Scientific (Waltham, MA). RGD peptide was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, sc-201176). Inhibitor of p38/MAPK signaling (SB 203580) was purchased from Cell Signaling (Danvers, Massachusetts). Inhibtor of NFκB signaling (Bay 11-7085) was purchased from Tocris (Minneapolis, MN).

Cells, culture conditions, and treatments

The murine cells J774.2 and RAW264.7 (hereto forth referred to as J774 and RAW, respectively), as well as mouse mammary carcinoma cells (4T1), fibroblasts (L929), and endothelial cells (2H11) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Red fluorescent protein (RFP)-expressing 4T1 cancer cells were obtained from Anticancer Inc. (San Diego, CA). Media and supplements were obtained from Hyclone (Logan, UT) unless noted. Cells were cultured at 37°C and 5% CO_2 in DMEM media supplemented with 10% fetal bovine serum (FBS; Atlanta Biologics, Atlanta, GA), gentamycin, and amphotericin B.

Bone marrow derived macrophages (BMDMs) were harvested from female Balb/C mice. Briefly, after mouse sacrifice, the femur and tibias were dissected. After a wash in 70% ethanol and phosphate buffered saline (PBS), the ends of each bone were cut and the bone marrow was flushed out with media. Cells were cultured in the

presence of L929 fibroblast conditioned media (20%) in supplemented media to promote macrophage differentiation for one week prior to experimental treatment (105, 106).

For all *in vitro* experiments, cells were seeded for 24 hours in FBS-supplemented media prior to starving and treatment in 0% FBS media. PBS was used as a vehicle control for most experiments. Experiments utilizing SB 203580 or Bay 11-7085 included a vehicle control containing an equivalent dilution of dimethyl sulfoxide (DMSO).

Orthotopic murine mammary cancer model

Female Balb/C mice (Jackson Lab, Bar Harbor, ME) were housed and maintained in the Vivarium in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of North Carolina at Charlotte. Mice were injected subcutaneously in an inguinal mammary fat pad with 3×10^5 RFP-expressing 4T1 cancer cells in 100 μl of PBS. Cancer cells were either injected alone, or supplemented with RAW macrophages (ratio 5 cancer cells: 1 macrophage). Macrophages were pre-treated with PBS vehicle control or periostin 10 μg/mL for 48 hours prior to co-injection into mice. Tumor growth was assessed by caliper measurements and fluorescence, measured as fluorescent radiant efficiency ([p/s/cm²/sr]/[μW/cm²]), over a 28-day period using an *in vivo* imaging system (IVIS, Perkin Elmer, Waltham, MA).

Enzyme-linked immunosorbent assays (ELISAs)

Concentrations of periostin, macrophage colony stimulating factor (MCSF), vascular endothelial growth factor (VEGF), transforming growth factor-beta 1 (TGF-

β1), interleukin-10 (IL-10), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in murine cell culture supernatants were assessed using ELISA kits (R&D Systems, Minneapolis, IN) according to the manufacturer‘s recommendations. The absorbance of each sample along with that of a standard curve was determined using a microplate reader (Biotek, Winooski, VT) and the concentrations of proteins (pg/mL) were derived from the standard curve.

Enzyme activity assays

Inducible nitric oxide synthase activity was determined by measuring nitrite production in culture supernatant. Briefly, supernatant was incubated with sulfanilimide for 10 minutes at room temperature to produce an intermediate compound. *N*-1-napthylethylenediamine dihydrochloride (NED) was added under acidic conditions for 10 minutes at room temperature, and the azo product was detected by colorimetric absorption at 550 nm. Nitrite concentration was derived from sodium nitrite standard curve.

Arginase 1 enzyme activity was assessed by activating arginase in the cell lysate for 10 minuntes at 55°C in the presence of magnesium chloride. L-arginine was added to samples to allow the production of urea for one hour. After termination of the reaction, α-isonitrosopropiophenone (ISPF) was added to react with urea. The colorimetric absorbance of the product was measured at 540 nm and the concentration was derived from a standard curve.

Proliferation assays

Macrophages were plated in 96 well-plates (40,000 cells/well) and treated with periostin 0-10 μg/mL for 24 to 48 hours. Macrophage proliferation was assessed using

both a sulforhodamine B (SRB) assay, and Hoechst cell counting. Briefly, the SRB assay consisted of cell incubation with 5% tricholoroacetic acid for 45 minutes at 37°C. Cells were washed twice with deionized water, and wells were air-dried. Cells were stained with 0.5% Sulforhadamine B (SRB) for 20 minutes at room temperature, then cells were washed with 1% acedic acid five times. Cells were air-dried, then 10nM Tris base (pH 10.5) was added. The absorbance was measured at 565 nm. For Hoechst cell counting, cells were stained with Hoechst vital nuclear dye, and the number of cells present was derived from a standard curve using a microplate reader.

Collection of conditioned media

Cells were plated in culture flasks for 24 hours in supplemented media. Cells were then starved for 24 hours, and the culture supernatant was collected (107). Non-adherent cells in the media were pelleted by centrifugation and saved. Adherent cells were removed with scraping and combined with the pelleted non-adherent cells. The number of viable cells from the flask per mL was assessed using Trypan blue staining and counting. Prior to use as treatments, conditioned media aliquots were filtered with a 0.2 μm sterile filter to prevent cellular-cross contamination. For experiments utilizing controlled amounts of conditioned media, the volume needed to equal 150,000 or 500,000 cells' worth of J774 conditioned media was calculated for treatment of 2.5 million cancer cells. This equates to approximately 6% or 20% of macrophages compared to the total number of cancer cells (tumor cells =100%).

Adhesion assay

Macrophages were stained with Hoechst nuclear stain (1:2000) for one hour then seeded at a density of 40,000 cells per well in a 96-well plate. Attachment of cells

was assessed by fluorescence reading after removing excess media and non-adherent cells. The number of attached cells was derived from a standard curve, and attachment was expressed as the percent of initial cells plated. Three seeding conditions were used to assess adhesion. In addition to uncoated tissue culture plates, plates were coated with periostin (20 ug/mL) or fibronectin (20 ug/mL) in PBS for 2 hours at room temperature.

Bead Phagocytosis Assay

Macrophages were stained with Hoechst nuclear dye (1:2000) for one hour and plated in a 96-well plate at a density of 40,000 cells per well in supplemented media. After 24 hours, the media was removed and replaced with serum-free media containing PBS or periostin 10.0 ug/mL. After treatment for 48 hours, cells were be incubated for 1-4 hours with red immunofluorescent polystyrene microspheres (1 μm diameter; Thermoscientific, Freemont, CA). Non-phagocytosed beads were removed and the plates were washed twice with PBS. The number of beads phagocytosed per cell was assessed using a fluorometer and derived from separate standard curves for beads and cells. Additionally, cells were detached and fixed in formalin, and the percent of phagocytic cells was quantified by flow-cytometry (Fortessa, BD Biosciences, San Jose, CA). For some experiments, RAW macrophages were pre-treated for two hours with vehicle control (VC), an inhibitor of integrin signaling (RGD) or an inhibitor of p38/MAPK (SB) prior to incubation with control PBS or periostin 10 μg/mL for 48 hours. For other experiments, an inhibitor of NFκB signaling (Bay 11-7085) was added at the end of the periostin incubation, four hours prior to incubation with polymer beads.

3.4 Results

Macrophage secretions increase cancer cell expression of periostin

First we assessed that macrophages are not a source of periostin in the microenvironment, consistent with previous studies (26). Indeed, macrophages did not secrete periostin, contrasting with cancer cells that secreted significantly higher baseline levels of periostin (Fig. 11A). Furthermore, macrophage secretion of periostin could not be induced by treatment with TGF-β1 (data not shown), a cytokine known to upregulate periostin expression in multiple cell types (70). Next, we tested the effect of macrophage conditioned media on cancer cell secretion of periostin. As shown Fig 11B, indeed, soluble secreted factors present in J774 conditioned media increased secretion of periostin by 4T1 cancer cells in a dose-dependent manner. None of the conditioned media collected from murine 2H11 endothelial cells, L929 fibroblasts, or 4T1 cancer cells affected 4T1 cancer cell secretion of periostin (data not shown). Furthermore, macrophage conditioned media did not affect endothelial cell and fibroblast expression of periostin expression (data not shown), supporting the specificity of this macrophage: cancer cell paracrine effect.

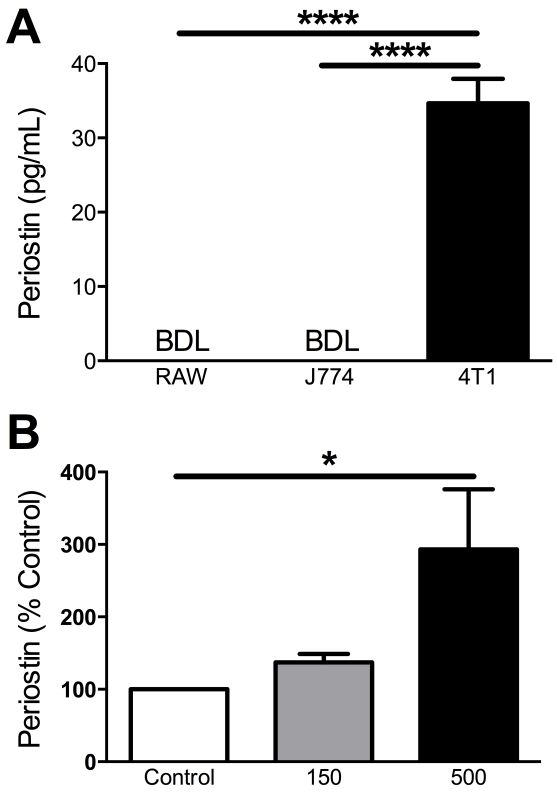


Figure 11: Macrophage secretions increase 4T1 cancer cell secretion of periostin. (A) RAW, J774, and 4T1 cancer cells were cultured for 24 hours, and periostin secretion was analyzed by ELISA. RAW and J774 macrophages secrete no periostin at baseline, while 4T1 cancer cells secrete higher baseline levels of periostin (ANOVA $p<0.0001$). ****$p<0.0001$. (B) Conditioned media was collected from J774 cells for 24 hours. 4T1 cancer cells were treated over 48 hours with increasing amounts of J774 conditioned media that would be equivalent to co-culture the presence of approximately 0, 6 and 20 percent macrophages per well (see Materials and Methods fro details). J774 conditioned media increased 4T1 secretion of periostin in a dose-dependent manner (ANOVA $p<0.0001$). *$p<0.05$.

Periostin alters VEGF and TGF- β1 secretion by RAW cells

Next, we investigated the effect of periostin on macrophage secretions as a surrogate for macrophage polarization and function (Fig. 12). Treatment of J774 macrophages with periostin (1 μg/mL for 48 hours) did not affect the secretion of macrophage colony stimulation factor (MCSF), vascular endothelial growth factor (VEGF), TGF- β1, interleukin-10 (IL-10), interleukin-6 (IL-6), or tumor necrosis factor-

α (TNF-α) (Fig. 12A-F). Interestingly, periostin significantly decreased RAW cell secretion of VEGF, an important pro-tumor growth factor, which promotes angiogenesis (Fig. 12H). Conversely, periostin increased secretion of TGF-β1, an important immunosuppressive cytokine (Fig. 12I). Periostin did not significantly affect RAW cell secretion of MCSF, IL-10, or IL-6 (Fig 12G, J-K).

Periostin does not affect macrophage polarization *in vitro*

To further assess the effect of periostin on macrophage polarization, macrophage iNOS and arginase activity were investigated (M1 and M2 markers, respectively)(Fig. 13). Treatment of un-stimulated J774 macrophages with periostin did not affect iNOS (Fig. 13A) or arginase activity (Fig. 13C). When macrophages were pre-stimulated for two hours with LPS to promote M1-like differentiation or PMA to promote M2-like differentiation prior to periostin treatment, iNOS and arginase activity were increased by LPS and PMA treatment as expected (Fig. 13B and Fig. 13D). However, periostin treatment did not lead to any additional change in enzyme activity compared to LPS or PMA treatment alone.

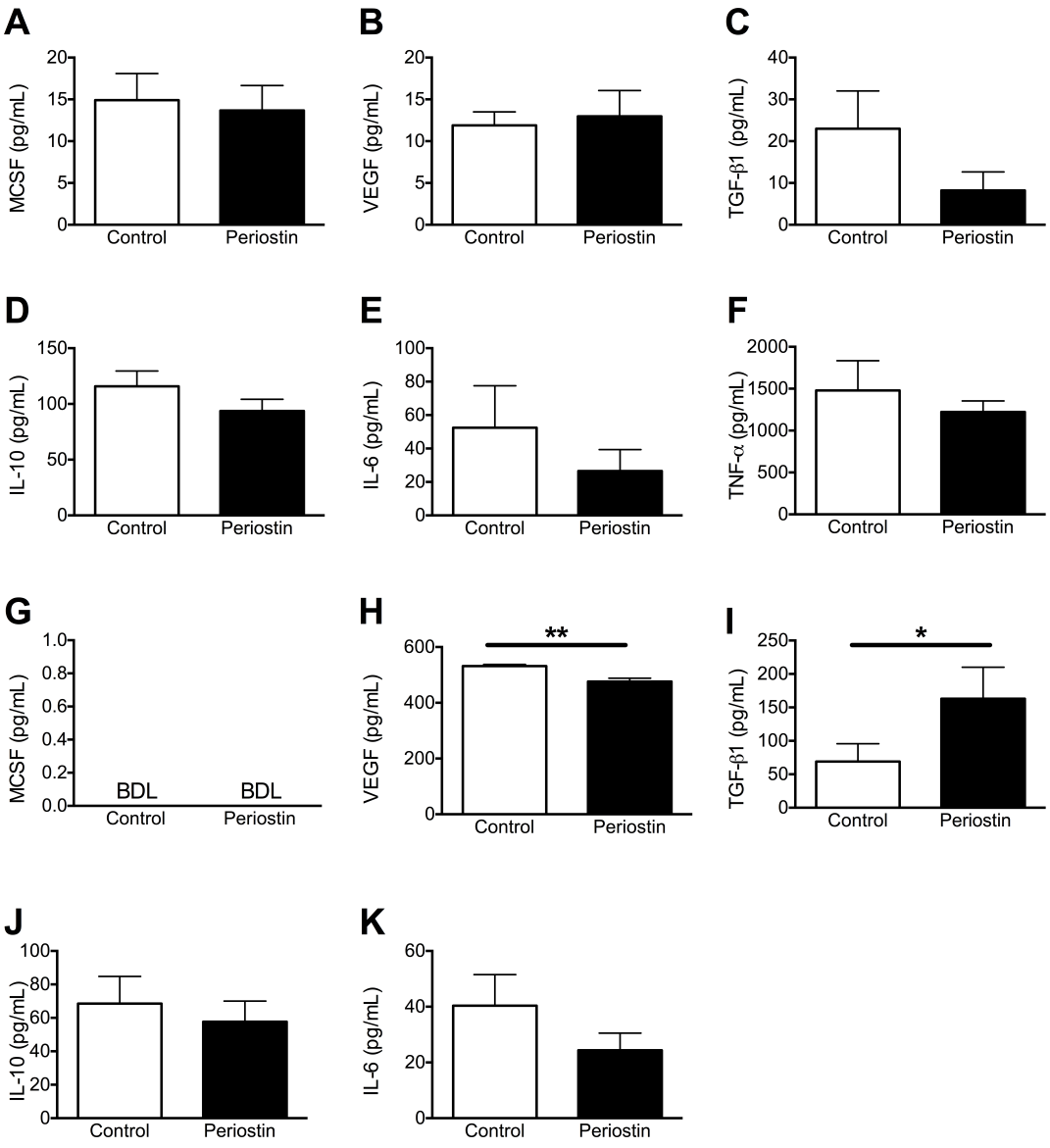


Figure 12: Periostin alters RAW cell secretion of VEGF and TGF-β1. RAW and J774 macrophages were treated with periostin 1.0 μg/mL for 48 hours and secreted cytokine levels were analyzed with ELISA. (A-F) Periostin treatment did not affect cytokine and growth factor secretions of J774 macrophages in the conditions tested. (G-K) Periostin decreased VEGF (H, student's t-test **$p<0.01$) and increased TGF-β1 cytokine secretion by RAW cells (I, paired t-test). *$p<0.05$.

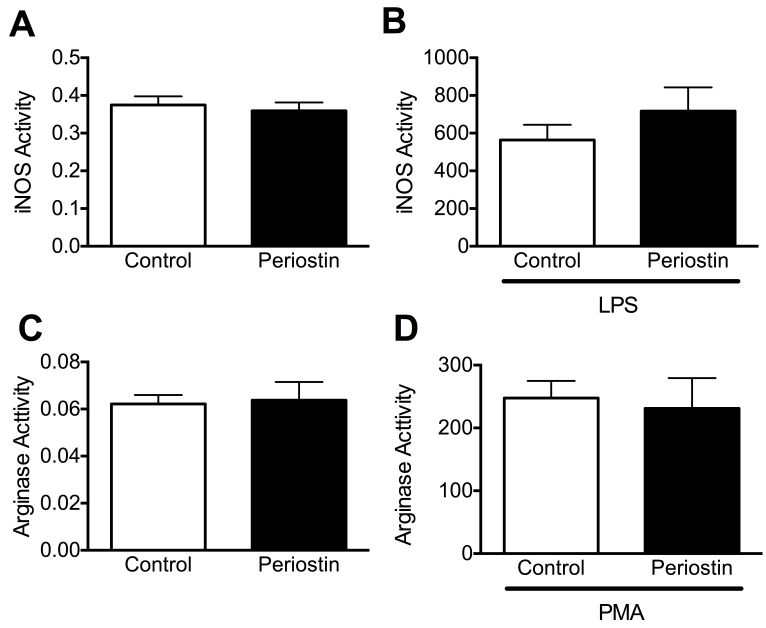


Figure 13: Periostin does not affect J774 and RAW macrophage polarization *in vitro*. (A and C) Un-stimulated J774 macrophages were treated for 48 hours with control PBS vs. periostin 1 μg/mL for 48 hours. Cells were scraped in the culture media, then were pelleted and washed. (B and D) Prior to periostin treatment, J774 macrophages were pre-treated with LPS (100 ng/mL) or PMA (320 nM) for 2 hours to stimulate the expression of M1 or M2 phenotypes, respectively. (A and B) iNOS activity was determined by measuring nitrite concentrations in culture supernatant. Periostin did not affect iNOS activity in either (A) un-stimulated macrophages (nitrite, μM) or (B) LPS-stimulated macrophages (nitrite, % of un-stimulated macrophages). (C and D) Arginase activity was determined in cell lysates. Periostin did not affect arginase activity of (C) un-stimulated macrophages (urea production, μg/mL) or (D) PMA-stimulated macrophages urea, % of un-stimulated macrophages).

Periostin does not affect macrophage viability in vitro

Periostin is known to increase proliferation of both cancer cells and mesenchymal cell types (15, 70). Thus, we next investigated the effect of periostin on macrophage viability (Fig. 14). Treatment with periostin 10 μg/mL for 24-48 hours did not affect the viability of RAW cells (Fig. 14A), J774 cells (Fig. 14B), or bone marrow-derived macrophages (Fig. 14C). Similarly, periostin did not affect survival of macrophages for up to 7 days (data not shown).

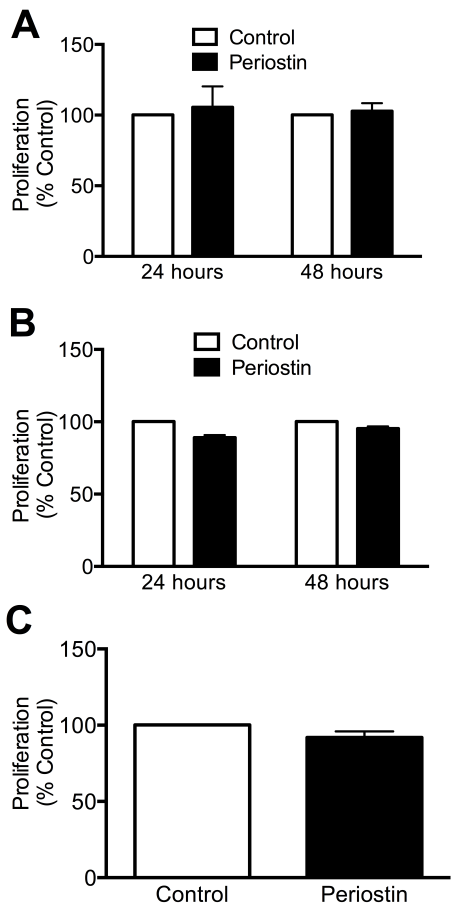


Figure 14: Periostin does not affect macrophage viability *in vitro*. Macrophages were cultured in the presence of periostin 10 μg/mL for 24 to 48 hours, and the percent of periostin-treated cells present was compared to the number of control cells using (A and B) an SRB assay or (C) Hoechst nuclear staining. Cell numbers were derived from standard curves and percentages of control are presented. Periostin had no effect on either (A) RAW or (B) J774 macrophage proliferation. (C) Periostin also had no effect on bone marrow-derived macrophage proliferation at 48 hours.

Periostin decreases macrophage attachment to fibronectin

Periostin was initially classified as an adhesion molecule, due to its promotion of ostebolast adhesion (2). Thus, we next investigated periostin's effect on macrophage adhesion under multiple conditions. First, periostin's effect on macrophage adhesion to tissue culture vessels was investigated. Periostin did not affect RAW or J774 cell attachment, regardless of dose (range 0.1-10 μg/mL). Second, tissue culture plates were

coated with periostin 10 μg/mL. RAW and J774 macrophages also did not adhere at a higher rate to periostin-coated plates compared to uncoated plates (data not shown). Interestingly, periostin significantly decreased adhesion of bone marrow-derived macrophages, RAW, and J774 cells to fibronectin-coated plates (Fig. 15).

Periostin inhibits macrophage phagocytosis of polymer beads

Non-specific phagocytosis is an import function of macrophages, which in the increases removal of debris in inflammatory tissues (71, 91). Therefore, we tested whether periostin affects non-specific phagocytosis of polymer beads by RAW, J774 and bone-marrow derived macrophages (Fig. 16). Treatment for 48 hours with periostin (10 μg/ml) led to a decrease in the percent of phagocytic cells regardless of the macrophage tested (Fig. 16 A-D). Furthermore, periostin significantly decreased the number of beads phagocytosed on average per cell in both RAW and J774 cells, but not of bone marrow-derived macrophages, which had a higher baseline phagocytic index (Fig. 16 E-G).

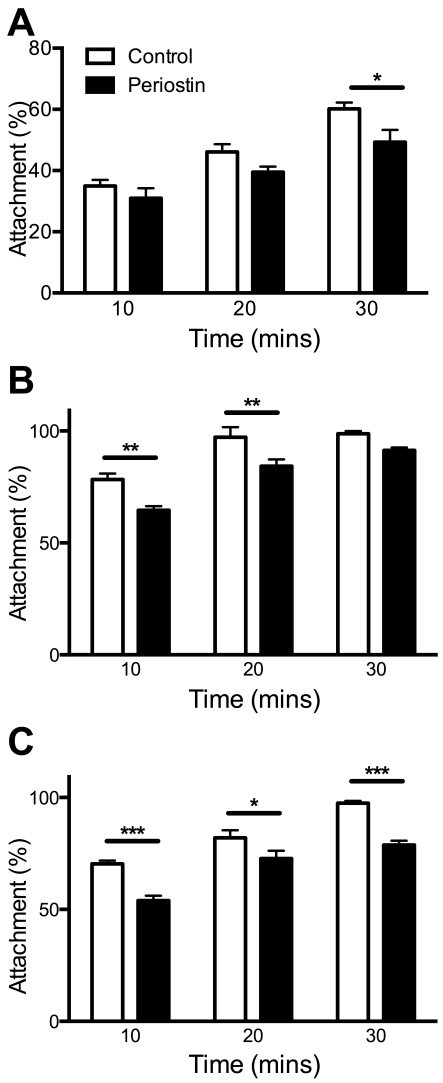


Figure 15: Periostin decreases macrophage attachment to fibronectin. Macrophages were mixed with PBS (control) or periostin 10 μg/mL then immediately seeded for 10 to 30 minutes onto fibronectin-coated plates. The percent of cells attached at each time point is presented. Periostin decreased attachment of (A) bone marrow macrophages (Two-way ANOVA, time $p<0.0001$, periostin treatment $p=0.0048$, $*p<0.05$), (B) RAW cells (Two-way ANOVA, time $p<0.0001$, periostin treatment $p<0.0001$, $**p<0.01$), and (C) J774 cells (Two way-ANOVA, time $p<0.0001$, periostin treatment $p<0.0001$, $*p<0.05$, $***p<0.001$).

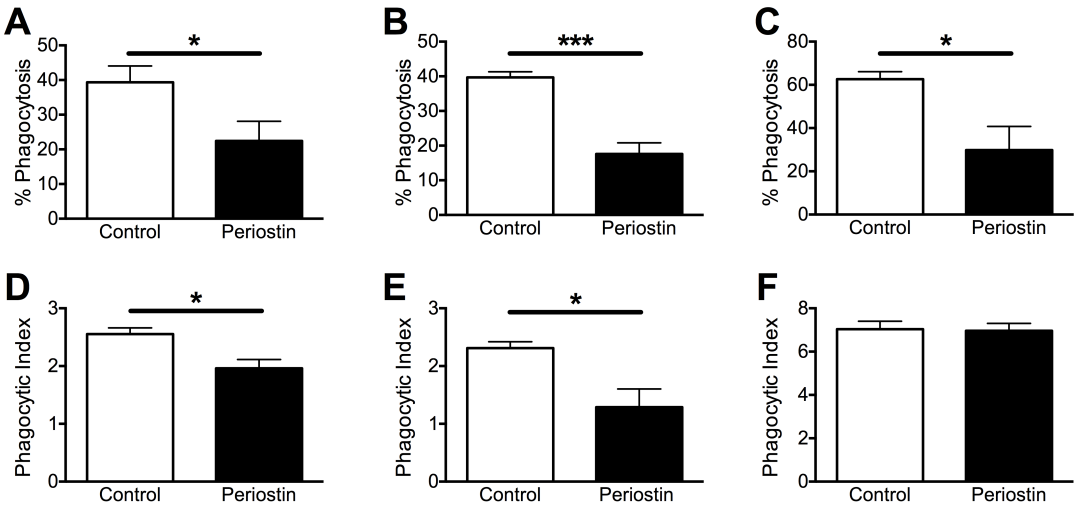


Figure 16: Periostin inhibits macrophage phagocytosis of polymer beads. Cells were treated with periostin 10 μg/mL for 48 hours then incubated with red fluorescent polymer beads for four hours. Periostin inhibited the phagocytosis of beads by (A) RAW cells, (B) J774 cells, and C) murine bone marrow-derived macrophages as assessed by flow-cytometry (% of cells which phagocytosed at least one bead, student's t-test *$p<0.05$, ***$p<0.001$). Periostin also decreased the phagocytic index (average number of beads phagocytosed per cell) of (D) RAW and (E) J774 cells, but not of (F) BMDMs which had a higher baseline phagocytic index (student's t-test *$p<0.05$).

Periostin inhibition of macrophage phagocytosis is not rescued by inhibitors of integrins, p38/MAPK, and NFκB signaling.

Next we aimed to investigate the mechanism by which periostin mediates downregulation of non-specific phagocytosis. Periostin is known to signal via integrins and to activate many downstream pathways including MAPK and NFκB. Both of these pathways are important in normal macrophage functioning, and therefore, we hypothesized that treatment with inhibitors of these pathways might rescue periostin's inhibition of phagocytosis (Fig. 17). Towards this end, RAW macrophages were pre-treated for two hours with vehicle control (VC), an inhibitor of integrin signaling (RGD) or an inhibitor of p38/MAPK (SB) prior to incubation with control PBS or periostin (10 μg/mL for 48 hours). Macrophages were incubated with red fluorescent

polymer beads for one hour, and the percent of phagocytic cells was assessed by flow-cytometry (Fig. 17A). In the conditions tested, integrin and MAPK inhibitors alone did not affect phagocytosis (white bars). Moreover, periostin led to similar decreases in bead phagocytosis, regardless of the inhibitor tested (black bars). To assess the role of NFκB signaling, RAW macrophages were treated with periostin (10 μg/mL for 48) hours, and an inhibitor of NFκB signaling was added during the final 4 hours of treatment (Fig. 17B). In those conditions, NFκB inhibition alone did not affect the phagocytosis of macrophages after one hour of incubation with polymer beads (white bars). Moreover, periostin decreased the phagocytosis regardless of NFκB inhibition (black bars).

Periostin pre-treated macrophages inhibit mammary tumor growth

Our *in vitro* studies suggested that periostin had a suppressive effect on macrophage functions. Therefore, we next aimed to investigate whether that effect translated in an anti-tumor effect *in vivo*. We assessed the specific effect of periostin on tumor-associated macrophages, using an immunocompetent orthotopic mammary cancer model and injecting 4T1-RFP-expressing cancer cells into a mammary fat of female, Balb/C mice (Fig. 18). Cancer cells were injected alone or in combination with RAW macrophages at a ratio of 5:1 cancer cells: macrophages. Co-injection of RAW cells led to decreased tumor size compared to 4T1 alone (Fig. 18A). Furthermore, periostin-pre-treatment of macrophages with periostin (10 μg/mL) led to further suppression of primary tumor growth. Interestingly, co-injection of macrophages, regardless of periostin pre-treatment did not affect distant metastasis under the conditions tested (Fig. 18B).

3.5 Discussion

Previous studies, including our own, have highlighted periostin as a tumor-promoting protein, with effects mediated through cancer cells, endothelial cells, and fibroblasts (15, 26, 108). However, here our data support an anti-tumor effect of periostin on macrophages in the tumor microenvironment *in vivo* and *in vitro*.

First, we confirmed that periostin is not secreted by macrophages, suggesting that periostin's effects on macrophages are dependent on periostin secretion in the tumor microenvironment by other cells such as cancer cells, fibroblasts, and endothelial cells (15, 26). Interestingly, we discovered that macrophages release soluble factors that act in a paracrine manner to increase cancer cell expression of periostin. As macrophage conditioned media did not elicit an increase in periostin secretion by fibroblasts or endothelial cells, nor did conditioned media from endothelial cells or fibroblasts elicit tumor periostin secretion, this suggests a specific paracrine communication between macrophages and tumor cells. Accordingly, these macrophage: cancer cell paracrine communications may modulate periostin concentration in the breast tumor microenvironment.

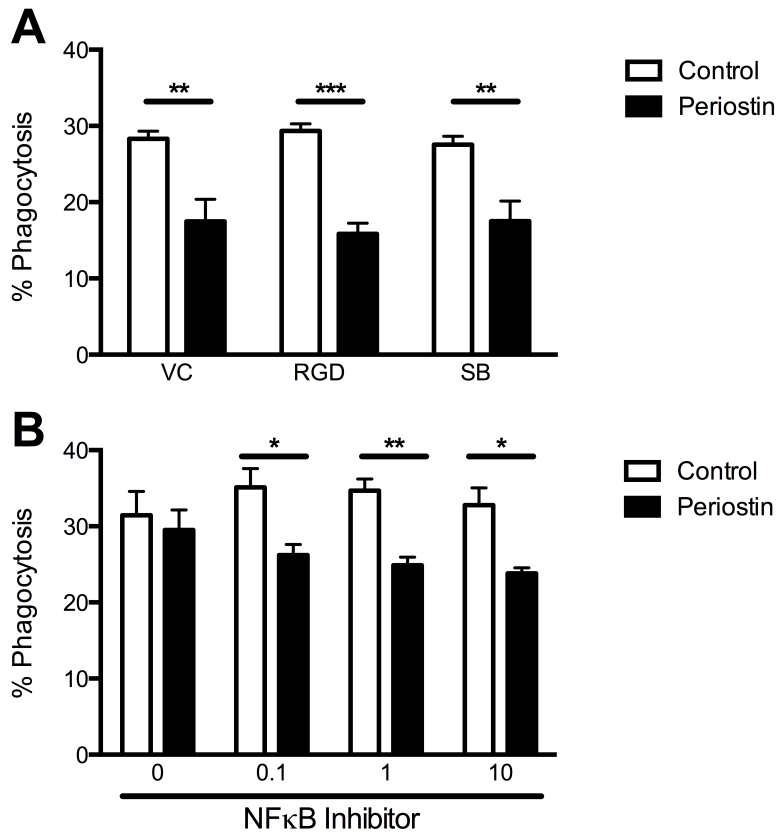


Figure 17: Periostin's inhbition of macrophage phagocytosis is not rescued by inhibitors of integrins, p38/MAPK, and NFκB signaling. (A) RAW macrophages were pre-treated for two hours with vehicle control (VC), an inhibitor of integrin signaling (RGD peptide 100 μM), or an inhibitor of p38/MAPK (SB 203580 1 μM) prior to incubation with control PBS or periostin 10 μg/mL for 48 hours. Macrophages were incubated with red fluorescent polymer beads for one hour, and the percent of phagocytic cells was assessed by flow-cytometry. Integrin and MAPK inhibitors alone did not affect phagocytosis (white bars) in the conditions and at the concentrations tested. Rather, incubation with periostin led to decreased phagocytosis, regardless of inhibition of those signaling pathways (black bars, two-way ANOVA, periostin treatment $p<0.0001$, inhibitor treatment N.S., $^{**}p<0.01$, $^{***}p<0.001$). (B) RAW macrophages were treated with periostin 10 μg/mL for 48 hours, and an inhibitor of NFκB signaling was added during the final 4 hours of treatment (Bay 11-7085, dose curve 0-10 uM). Macrophages were then incubated with polymer beads for one hour and phagocytosis was assessed by flow-cytometry. NFκB inhibition alone did not affect % phagocytosis (white bars). Regardless of NFκB inhibition, incubation with periostin decreased the percent of phagocytosis (black bars, two-way ANOVA, periostin treatment $p<0.0001$, inhibitor treatment N.S., $^{*}p<0.05$, $^{**}p<0.01$).

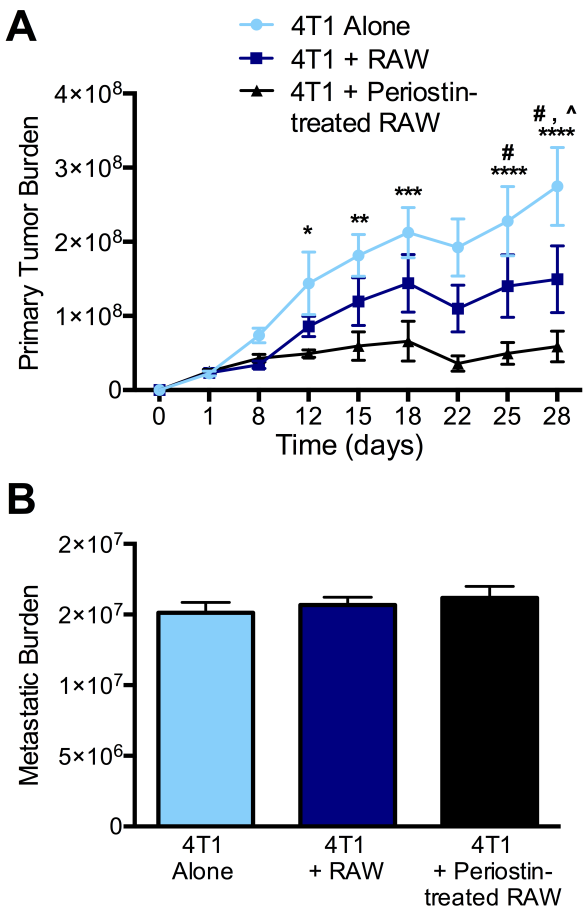


Figure 18: Periostin pre-treated macrophages inhibit mammary tumor progression. 4T1-RFP cells were subcutaneously injected in female Balb/C mice either alone (light blue circle) or in combination with RAW cells in a 5:1 ratio of cancer cells: macrophages. Macrophages were pre-treated with PBS vehicle control (dark blue square) or periostin 10 µg/mL (black triangle) for 48 hours prior to co-injection into mice. Primary tumor size and metastasis was assessed by fluorescence intensity measurement. (A) Co-injection of RAW macrophages led to decreased tumor size compared to 4T1 cells alone. Pre-incubation of macrophages with periostin led to a further decrease in tumor size (two-way repeated measures ANOVA, time $p<0.0001$, treatment $p<0.0001$, interaction $p= 0.0061$; 4T1 alone vs. 4T1 + Periostin-treated RAW *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$; 4T1 + RAW vs. 4T1 + Periostin-treated RAW #$p<0.05$; 4T1 alone vs. 4T1 + RAW ^$p<0.01$). (B) These treatments did lead to changes in distant metastasis.

Our data also indicate that periostin decreases RAW cell secretion of VEGF. VEGF is a key promoter of angiogenesis, which in the tumor microenvironment promotes both tumor growth and dissemination (109). Decreasing VEGF expression or

signaling is anti-tumoral and is the basis anti-angiogenic cancer therapy (109, 110). Contrasting with the decrease of macrophage VEGF expression stimulated by periostin, periostin has been shown to increase cancer cell secretion of VEGF and to have direct effects on endothelial cell proliferation (25) highlighting the complexity of periostin's effects within the tumor microenvironment.

Periostin treatment also led to increased TGF- β1 secretion by macrophages. We have previously shown that periostin increases TGF- β1 secretion by cancer cells, as part of an autocrine feedback loop (Thesis Chapter 2). This loop appears to be conserved in RAW cells and may contribute to additional periostin secretion in the tumor microenvironment.

Interestingly, periostin decreased macrophage non-specific phagocytosis *in vitro*. Unlike antibody-mediated and complement-mediated phagocytosis, non-specific phagocytosis is poorly understood. However, integrin binding may play a role (99, 111). Non-specific phagocytosis of debris and extracellular matrix is a critical function of macrophages as part of the innate immune system (85), particularly playing a role during the early inflammatory stages of the wound healing response (71). Here, periostin inhibited macrophage non-specific phagocytosis, which does not confer a decidedly pro-or anti-tumor effect. However, breakdown of the ECM by macrophages is thought to facilitate breast cancer invasion in vivo (85, 91). The mechanism by which periostin exerts its effect on macrophage phagocytosis remains elusive. Indeed, blocking integrins with the RGD peptide did not alter periostin's effects in the conditions tested. Likewise, inhibition of the p38/MAPK and NFκB signaling pathways did not rescue periostin's effect on macrophage phagocytosis.

The fact that periostin's effects are mainly observed after chronic treatment is of interest. With the exception of the adhesion assay, in our experiments, periostin's effects were most pronounced at 48 hours, but required at least 18-24 hours of treatment (data not shown). This observation suggests that periostin's effects may involve multiple signaling pathways that reorganize cellular function.

Our data also highlighted that periostin decreased macrophage attachment to fibronectin. This effect did not require prolonged incubation, suggesting a physical blocking of adhesion. Periostin's emilin (EMI) domain allows periostin to bind to collagen I and fibronectin. Periostin is also known to play a role in the assembly of fibronectin and tenascin-C hexabrachion structures/scaffolds (112). Interestingly, periostin also decreases adhesion of 4T1 breast cancer cells (33) but increases eosinophil attachment to fibronectin *in vitro* (95). Given these prior findings, periostin's role in decreasing macrophage attachment to fibronectin appears to be specific to macrophage-lineage cells. Because periostin and fibronectin both bind to integrins, there are at least two possible mechanisms for this immediate physical competition. First, periostin may outcompete fibronectin for integrin binding on the macrophage cell surface. Second, periostin's affinity for binding fibronectin rather than macrophage integrins could prevent macrophages from binding to fibronectin directly. As in our experiments periostin-coating of plates did not increase macrophage adhesion, periostin's effect is likely due to blocking of fibronectin's binding sites preventing direct macrophage adhesion. Consistent with those findings, Zhou et al. recently demonstrated that periostin promotes macrophage migration *in vitro* (113).

Finally, periostin-treated tumor-associated macrophages led to a significant reduction in primary tumor size in the *in vivo* immunocompetent orthotopic 4T1 mammary cancer model. Macrophages can function in a pro-tumor or anti-tumor capacity *in vivo* depending on the local environment (85, 91), and here the periostin-treated macrophages significantly limited tumor progression. This growth suppression may be related to the effects seen *in vitro*, for example due to decreased macrophage secretion of VEGF and decreased ability to phagocytose the ECM to promote cancer cell local invasion.

Given the plasticity of macrophages, the long-lasting effects associated with periostin pre-treatment are surprising. Nevertheless, the observations made here underline the therapeutic promise in periostin pre-programmed macrophages. Additional investigation is warranted to uncover the mechanisms involved in these sustained inhibitory effects on mammary tumor growth.

Interestingly, untreated RAW macrophages also decreased primary tumor size over size. This suggests that the RAW macrophages used might have been pre-polarized towards an M1-like phenotype prior to injection, and 4T1 cancer cells were unable to effectively re-polarize them towards a tumor-supportive M2 phenotype during the course of the study.

By design, our study bypassed the effects of periostin on other cell types, allowing the investigation of periostin's specific effect on macrophages. Based on our results, we speculate that the infiltration of macrophages into a tumor may lead to local upregulation of periostin expression by cancer cells through secreted TGF-β1 and likely other soluble factors. High levels of periostin actually decrease macrophage

phagocytosis, ECM remodeling, and VEGF secretion, all of which represent key functions of tumor-associated macrophages in the cancer microenvironment. In the tumor bed, however, periostin upregulation simultaneously affects other cells present in the microenvironment, overcoming periostin's specific effects on macrophages and causing an overall pro-tumor effect. This study highlights the complexity and importance of ECM protein-cellular interactions and provides new insight into periostin as an immunoregulatory molecule.

CHAPTER 4: OUTCOMES IN THE MANAGEMENT OF ADULT SOFT TISSUE SARCOMAS

4.1 Abstract

Adult soft tissue sarcomas (STSs) are heterogeneous neoplasms that account for 11,410 new diagnoses and 4, 390 deaths per year. This article summarizes recent NCCN guidelines for diagnosis and management of STSs of the extremities and retroperitoneum, as well as gastrointestinal stromal tumors (GIST). AJCC staging and recently reported NCDB data regarding outcomes are reviewed. Currently accepted STS prognostic variables are presented, as are future directions regarding the utility of molecular prognosticators and nomograms.

4.2 Introduction

Soft tissue sarcomas (STSs) are a heterogeneous group of solid neoplasms of mesenchymal cell origin arising from fat, muscle, fibrous connective tissue, vascular tissue, peripheral neural tissue, and visceral tissue. STSs represent less than 1% of newly diagnosed cancers in adults (114) and approximately 6% of newly diagnosed childhood malignancies (115). STSs account for at least 11,410 new diagnoses and 4,390 deaths per year (114); however the true incidence of STSs is currently underestimated because a larger number of patients with gastrointestinal stromal tumors (GISTs) may not have been included in tumor registry databases before 2001. In the

United States, the incidence of GISTs is expected to be approximately 3,000 cases per year (116).

More than 50 different histologic subtypes of STS have been described, with the most common being undifferentiated pleomorphic sarcoma (previously known as malignant fibrous histiocytoma), liposarcoma, leiomyosarcoma, synovial sarcoma, and GIST (115, 117). The anatomic location of the primary tumor affects treatment and outcomes. In one series of 1240 patients with STS (excluding GIST), the most common primary sites were the extremities (59%), trunk (19%), internal trunk (intra-abdominal, retroperitoneal, and pelvis, 15%), and head and neck (6%)(117). Approximately 10% of patients with STS present with metastatic disease, with the most common location being the lungs (118-120).

As stated in the American Joint Committee on Cancer (AJCC) 7th edition, staging provides patients and their physicians the critical benchmark for defining prognosis and the likelihood of overcoming cancer, for determining the best treatment approach, and for defining groups for inclusion in clinical trials (121). The level of data supporting the staging systems varies among disease sites; particularly for the less common cancers such as sarcomas, less outcome data is available.

Soft tissue sarcoma outcomes are reported in smaller numbers as case series, and in more robust numbers to national databases, such as the National Cancer Data Base (NCDB) in North America and the European Organisation for Research and Treatment of Cancer (EORTC) in Europe. Common endpoints regardless of therapy include overall survival and disease free survival. In addition to survival outcomes, meaningful outcomes in the surgical treatment of sarcomas include local recurrence, wound healing,

and infection rate. In the setting of limb salvage surgery, rate of limb salvage or amputation are reported. Additionally, outcomes following radiation therapy are reported to include lymphedema, fibrosis and stiffness. Pathologic fractures in irradiated bone and secondary malignancies in irradiated fields have also been reported in small case series.

Soft tissue sarcoma outcomes are determined by a multitude of factors and decision points. Patient-specific and tumor-specific factors have been well described to impact outcomes, including patient age (122, 123), marital status (124), tumor size (119, 122, 125-127), depth (126), histologic grade (117, 119, 126), microvascular invasion (117), tumor location (121, 127), and presence or absence of lymph node involvement and/or metastasis (121, 128, 129). Factors intrinsic to clinical care have also been shown to impact outcomes, in particular the involvement of a multidisciplinary team with expertise in sarcoma management prior to commencement of local therapy (130, 131), appropriate biopsy planning and placement (132), appropriate surgical resection with negative margins (132), and use of adjuvant local or systemic therapies as appropriate (132, 133).

The goals of this paper are to 1) summarize recent guidelines for management and staging of soft tissue sarcomas of the extremities , retroperitoneum and gastrointestinal stromal tumors (GIST), 2) review recently reported STS outcomes, and 3) discuss future directions in sarcoma care.

4.3 Extremity Soft Tissue Sarcoma

Initial Workup

According to National Comprehensive Cancer Network (NCCN) Guidelines, all STS patients should be evaluated and managed by a multidisciplinary team with expertise and experience in sarcoma, prior to the initiation of therapy (134)(Figure 19). The initial workup for an extremity sarcoma begins with a history and physical exam, followed by imaging of the primary tumor. A carefully planned biopsy should be performed by an experienced surgeon (or radiologist) with the intent to obtain a histologic diagnosis and allow tumor grading by an experienced pathologist. Ideally, the initial biopsy is carefully planned so that the biopsy site may be excised en bloc with the definitive surgical specimen, particularly should an open biopsy be performed. Generally a core needle or incisional biopsy is preferred; however in selected institutions with clinical and cytopathologic expertise, a fine needle aspiration may be acceptable (135, 136). Chest imaging should be obtained for staging purposes (118), and additional body imaging such as PET may be considered to evaluate for metastasis (137).

Staging and Survival Prediction

Extremity sarcomas are staged according to current AJCC 7th edition guidelines, which include tumor size, depth, nodal involvement, metastasis, and histologic grade (121) (Table 2). 5-year overall survival for soft tissue sarcomas is approximately 57% (138). AJCC stage is predictive of overall survival (139) (Figure 20), and is utilized in the NCCN Guidelines for STS management. Comprehensive grading of soft tissue sarcomas is strongly correlated with disease-specific survival. The most widely used

grading system is the French Federation of Cancer Centers Sarcoma Group (FNCLCC) system, which is based on tumor differentiation, mitosis count, and tumor necrosis (140). A comparison of the earlier AJCC grading system to the currently recommended FNCLCC in a cohort of 410 patients with STS revealed that the FNCLCC system may have a slightly increased ability to predict distant metastasis development and survival (140).

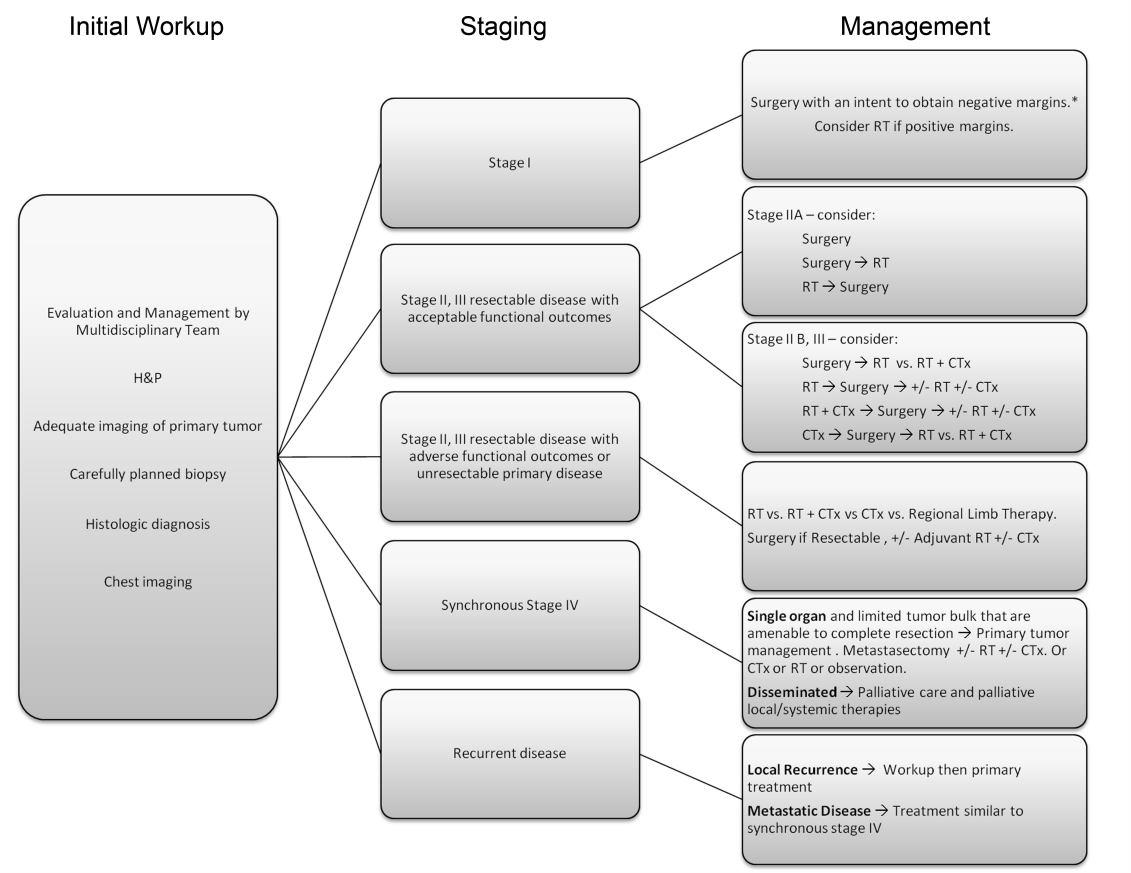


Figure 19: Overview of NCCN guidelines for management of extremity soft tissue sarcomas, not including rhabdomyosarcoma or desmoid tumors. RT – radiation therapy, CTx – chemotherapy. *Final margins > 1.0 cm or intact fascial plane.

Table 2: AJCC staging of soft tissue sarcomas*				
Primary Tumor (T)				
TX	Primary tumor cannot be assessed			
T0	No evidence of primary tumor			
T1	Tumor ≤ 5 cm in greatest dimension			
T1a	Superficial tumor			
T1b	Deep tumor			
T2	Tumor > 5 cm in greatest dimension			
T2a	Superficial tumor			
T2b	Deep tumor			
Regional Lymph Nodes (N)				
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph node metastasis			
N1	Regional lymph node metastasis			
Distant Metastasis (M)				
M0	No distant metastasis			
M1	Distant metastasis			
Histologic Grade (G)				
GX	Grade cannot be assessed			
G1	Well differentiated			
G2	Moderately differentiated			
G3	Poorly differentiated			
Anatomic Stage/Prognostic Groups				
Stage IA	T1a	N0	M0	G1, GX
	T1b	N0	M0	G1, GX
Stage IB	T2a	N0	M0	G1, GX
	T2b	N0	M0	G1, GX
Stage IIA	T1a	N0	M0	G2, G3
	T1b	N0	M0	G2, G3
Stage IIB	T2a	N0	M0	G2
	T2b	N0	M0	G2
Stage III	T2a, T2b	N0	M0	G3
	Any T	N1	M0	Any G
Stage IV	Any T	Any N	M1	Any G

*Reprinted with permission from AJCC 7th Ed.

To further aid in treatment decisions several nomograms, or graphical representations of statistical models, have been developed. These include models which

predict risk for death following surgical treatment for primary nonmetastatic STS (141)(Figure 21) and risk for death following local recurrence (142) . These nomograms are available for free use online by patients and physicians (143). More recently, a nomogram calculating risk for local recurrence after surgical treatment has also been developed to help access the need for postoperative radiotherapy (144). Furthermore, histology-specific nomograms have been developed to predict disease specific survival for liposarcoma (145) and synovial sarcoma (146).

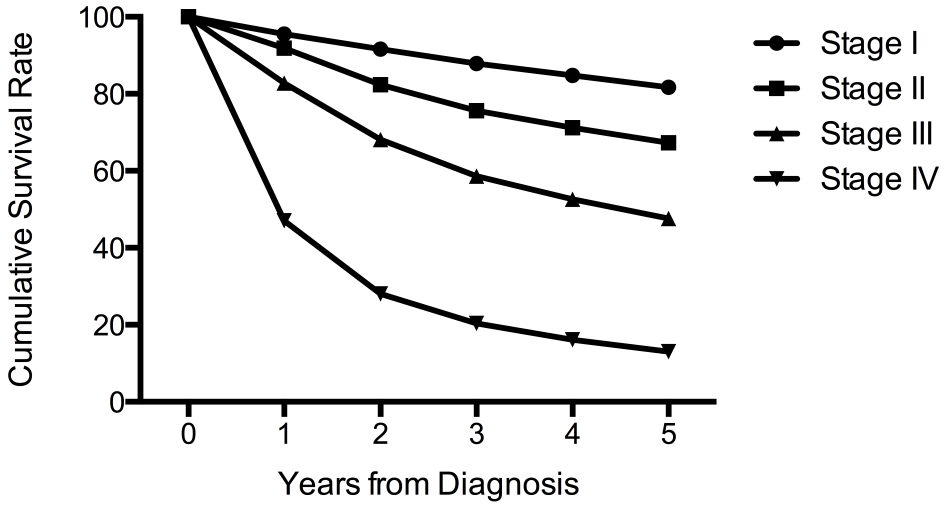


Figure 20: National Cancer Database (NCDB) observed national survival data for soft tissue sarcoma 2003-2006, including 1,334 programs and 14,811 patients.

Surgery

Surgical tumor resection with intent to obtain negative histologic margins (R0 resection) is a mainstay in management of the majority of STSs (134). A recent review and guideline development for the Ontario Sarcoma Disease Site Group presented a meta-analysis of surgical margin guidelines for soft tissue sarcoma within the MEDLINE and EMBASE databases as well as the Cochrane Library (132). Their

recommendation was: “In limb salvage surgery for STS, the operation should be planned with the objective of obtaining a clear margin. However, to preserve functionality, surgery may result in a close or even a microscopically positive margin. Based upon the consensus opinion of an expert panel, a close margin is to be considered less than one cm after formalin fixation. In the circumstance of a close or microscopically positive margin, the use of preoperative or postoperative radiation may be considered.” Close margins may be necessary to preserve uninvolved critical neurovascular structures, bones, and joints, and to preserve functionality. Final margins greater than 1.0 cm or an intact fascial plane are preferred, as some authors report positive surgical margins are associated with a higher rate of local recurrence and a decreased rate of survival (125, 147-152). Radical resection is not routinely necessary, and limb preservation is preferred if it is possible in the setting of an appropriate oncologic resection. If closed suction drainage is used, the drains should be placed close to the edge of the surgical incision (in case re-resection or radiation is indicated). In the setting of positive margins (R1 resection), surgical re-resection to obtain negative margins should be strongly considered, weighing potential clinical and functional outcomes. Amputation should be considered for patient preference, if gross total resection of the tumor is expected to render the limb nonfunctional, or when all limb-sparing options have been exhausted.

Radiation Therapy

In studies comparing adjuvant versus neoadjuvant radiation, there were no differences in local recurrence, distant recurrence, or progression free survival (147, 153). Potential advantages of preoperative radiation therapy (RT) include smaller

radiation field, lower radiation dose, and potential for tumor regression prior to surgical therapy. While preoperative RT has a higher rate of initial wound complications (153), it is associated with a lower rate of long-term morbidity (147). Postoperative RT has the advantage of reduced wound complications (153) however a larger volume of issue is typically irradiated postoperatively and to a higher final dose. Long term complications such as lymphedema and fibrosis rates are also greater for patients irradiated postoperatively (147).

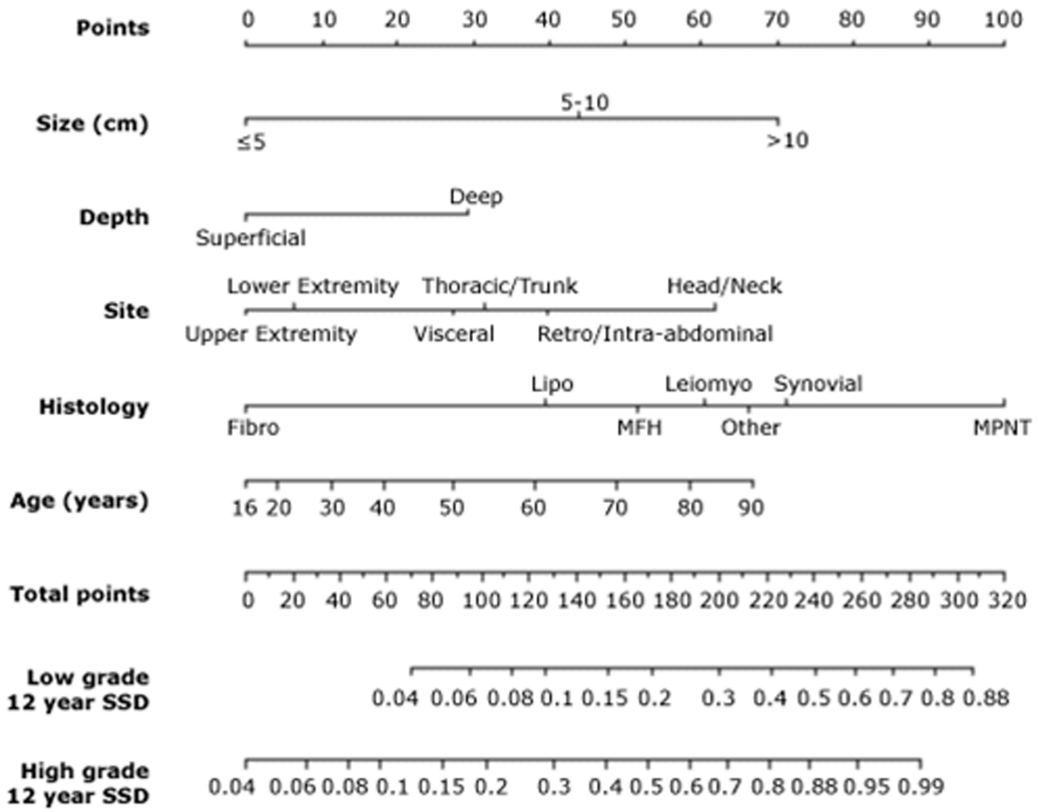


Figure 21: Postoperative nomogram for calculation of 12-year sarcoma –specific death (Kattan et al. 2002).

Adjuvant RT has been reported to improve local recurrence rates in patients with STS who undergo limb-sparing surgery, however it does not affect overall survival compared to no RT (122, 149, 154). RT recommendations should be made in the context of a multidisciplinary setting, as indications for RT may vary from patient to patient based upon AJCC stage. While patients with primary STS which are small in size (T1) and superficial may be treated with R0 resection alone (155), patients with large, deep, high-grade tumors and those with positive surgical margins (R1) have lower recurrence rates with postoperative RT compared to no RT (122). NCCN Guidelines recommend consideration for adjuvant RT in patients with a close soft tissue margin or a microscopically positive margin on bone, major blood vessels, or a major nerve. In addition, postoperative RT should be considered in patients at high risk for local recurrence including those with older age and stage III disease (123). The risk for local recurrence after surgical treatment can be calculated using a recently developed nomogram (144).

Chemotherapy

Adjuvant or neoadjuvant chemotherapy may be considered for some STS. Current NCCN guidelines include consideration of chemotherapy for tumors AJCC stage IIB or higher (134). While STSs are considered relatively chemoresistant, there is evidence that certain histologies are more chemosensitive to certain regimens, including leiomyosarcoma (156, 157), angiosarcoma (158), and synovial sarcoma (146, 159, 160). In these circumstances chemotherapy may be considered in a neoadjuvant setting to potentially reduce the size of a STS to improve local control surgical options and potentially allow for limb salvage.

There is conflicting data regarding the survival benefit of chemotherapy for adult STS. A meta-analysis of 14 randomized controlled trials (RCTs) composed of 1,568 patients with localized resectable extremity and nonextremity STSs revealed a significant effect on both local recurrence and relapse-free survival with doxorubicin-based chemotherapy versus no chemotherapy, with no significant effect on overall survival (161, 162). These findings equate to an absolute benefit of a 6% decrease in local recurrence and a 10% increase in relapse-free survival at 10 years. Since that time, an updated meta-analysis was performed which included four more RCTs, representing a total of 1,953 patients (133). This study revealed a statistically significant reduction in both local and distant recurrence, as well as an increase in overall survival, representing an absolute risk reduction of 6%. To further complicate this discussion, the recently conducted largest RCT to date comprised of 351 patients did not show a survival benefit with chemotherapy (163).

Some recent trials of combined neoadjuvant chemotherapy and radiation have shown promising results, including an impact on overall survival (164), and improvement of local control in high risk patients with positive margins following resection (148). At this point there are not sufficiently convincing data to recommend a standard regimen for all patents.

Fluorodeoxyglucose (FDG) positron emission tomography (PET) is a promising biomarker to indicate response to neoadjuvant chemotherapy in many tumor types (165). Schuetze et al. demonstrated the utility of FDG-PET in predicting outcomes of 46 patients with high-grade localized extremity soft tissue sarcomas after completion of neoadjuvant chemotherapy (137). In this study, a decline in SUVmax post-therapy of ≥

40% was associated with decreased risk for recurrence and death. Furthermore, in a recently published prospective trial including a cohort of 65 adults and children with bone or soft tissue sarcoma, change in maximum SUV uptake of FDG by tumors at mid-therapy point when compared to pre therapy SUV values was prognostic for progression-free survival and overall survival (166). Subanalysis for soft tissue sarcomas alone was not reported, however the potential for the utilization of FDG-PET to aid in directing systemic therapies, as well as prognostication, remains promising.

4.4 Retroperitoneal Sarcoma

Initial Workup

Retroperitoneal sarcomas (RPS) account for 15% of all sarcomas. As is the case with extremity STS, all patients with RPS should be evaluated and managed by an experienced sarcoma multidisciplinary team (Figure 22)(134). Initial history and physical examination should focus not only on symptoms pertinent to the mass effect of the RPS but also to those that might be indicative of other malignancies in the differential diagnosis of a retroperitoneal mass. RPS may present with non-specific abdominal symptoms often due to the large size of the tumor. Median size at presentation is 15-20 cm (167, 168).

CT of the abdomen and pelvis (or in some cases, MRI) is obtained to evaluate the extent of the mass, and a chest CT is obtained to rule out metastatic disease. Distinguishing the full extent of well-differentiated liposarcoma from surrounding retroperitoneal fat in particular may be challenging, emphasizing the importance of an experienced multidisciplinary team.

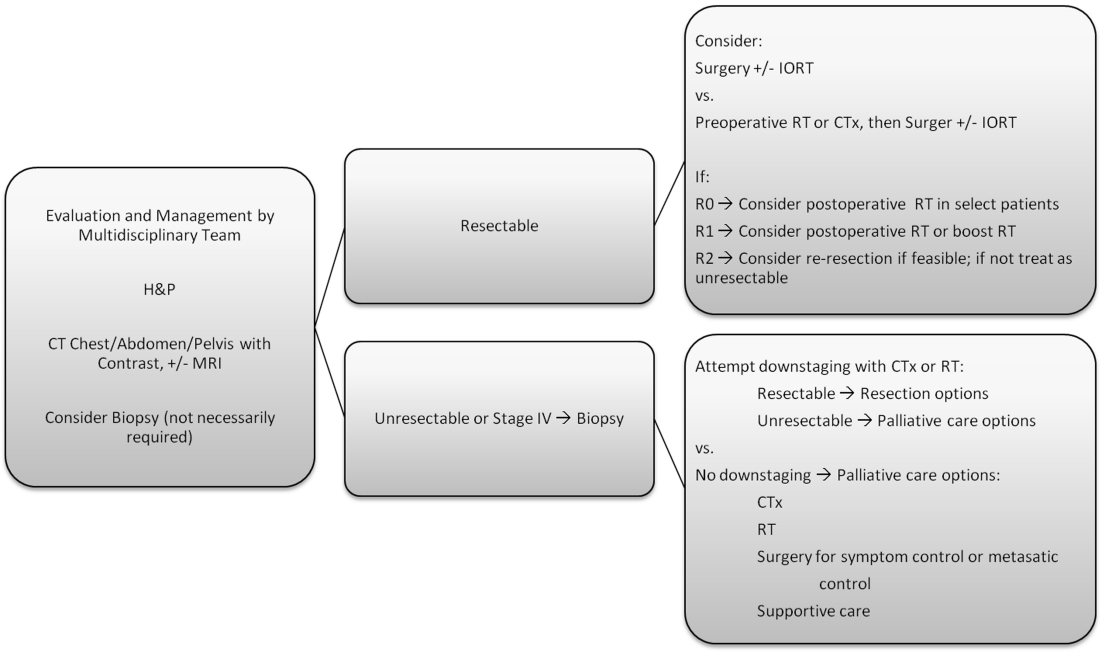


Figure 22: Overview of NCCN guidelines for management of retroperitoneal/intra-abdominal soft tissue sarcomas, not including GIST, desmoid tumors, or rhabdomyosarcoma. IORT – intraoperative radiation therapy; RT – radiation therapy, CTx – chemotherapy.

An image-guided biopsy is recommended, though not absolutely necessary. Biopsy is required if considering any preoperative therapy. Liposarcomas of the retroperitoneum with both well-differentiated and de-differentiated components have a characteristic appearance, and biopsy may not be necessary when evaluated by experienced teams. Core needle biopsies are preferred over fine-needle aspiration, since the latter rarely distinguish histologic subtypes of sarcoma. The most common RPS histologic subtypes are well-differentiated liposarcoma, dedifferentiated liposarcoma (with or without associated well-differentiated liposarcoma), and leiomyosarcoma (169, 170). The natural histories and patterns of failures for each are unique and should be factored into treatment planning. Pure well-differentiated liposarcoma only recurs locally (171, 172). Dedifferentiated liposarcoma has a high

rate of local recurrence, upwards of 80% and a low rate of distant metastases (167). In contrast, leiomyosarcoma predominantly metastasizes hematogenously and has a lower rate of local recurrence (171).

Staging and Survival Prediction

As with extremity STS, RPS are staged according to the AJCC staging system (Table 2)(Figure 23). However, a critical short-coming of the current AJCC staging system is that anatomic site and histologic subtype are not incorporated. In recent years, nomograms have been developed which are more specific to both site and histology (Figure 24)(145, 171, 173). They incorporate independent predictors specific to RPS and may include treatment factors.

Retrospective comparisons of series collected from prospectively maintained databases have demonstrated 5-year local control rates of 40% to 80% and 5-year overall survival rates of 50% to 70% (168, 172, 174-178).

Surgery

Surgery remains the only potentially curative treatment. The goal of therapy is a macroscopically complete resection, but because of the large size and anatomic complexity of RPS, true R0 resections (negative microscopic margins) are rare. A macroscopically incomplete resection should be avoided, as outcomes after R2 resection are no better than for patients with unresectable RPS (179, 180).

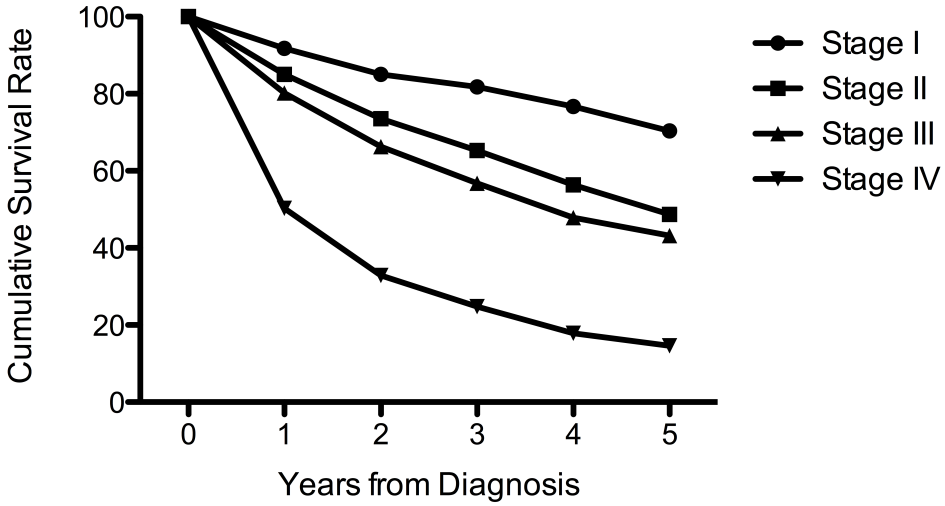


Figure 23: National Cancer Database (NCDB) observed national survival data for retroperitoneal tumors 2003-2006, including 654 programs and 1,674 patients.

Since each individual patient's presentation is unique, thorough review of the imaging is essential. While RPS do not usually invade adjacent organs, contiguous organ resection is commonly required. Surrounding organs are often adherent to or encased by the tumor, or their vascular supply is involved. Ipsilateral colectomy (with mesocolon serving as the anterior margin of the retroperitoneal compartment) and ipsilateral nephrectomy are commonly required (181, 182). Contralateral renal function should be assessed with a imaging such as a split function renal scan if an ipsilateral nephrectomy is planned. Distal pancreatectomy and splenectomy may be required for higher left-sided RPS, even without overt invasion. However, pancreaticoduodenectomy and right hepatectomy are usually reserved for invasive right-sided lesions due to higher potential morbidity. Preservation of specific organs should be considered on an individual basis. Deciding which neurovascular structures

to sacrifice or preserve requires weighing the potential for local control against the potential for long-term dysfunction.

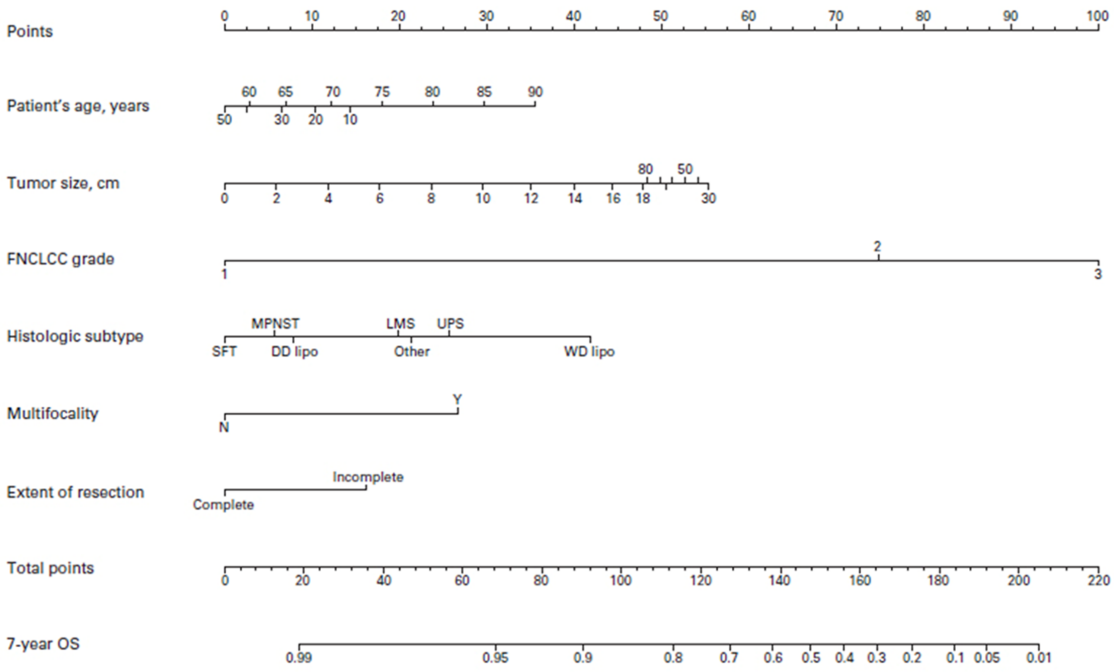


Figure 24: Postoperative nomogram for calculation of 7-year overall survival in patients with retroperitoneal soft tissue sarcoma (Gronchi et al 2013). Histologic subtypes - DD lipo, dedifferentiated liposarcoma; LMS, leiomyosarcoma; MPNST, malignant peripheral nerve sheath tumor; SFT, solitary fibrous tumor; UPS, undifferentiated pleomorphic sarcoma; WD lipo, well-differentiated liposarcoma.

Radiation

The role of radiation therapy has been evaluated in several small prospective trials and single institution studies. In a study of intraoperative radiation therapy (IORT) plus postoperative external beam radiation therapy (EBRT) versus postoperative EBRT alone, the addition of IORT reduced the rate of local recurrence but did not improve survival, and it was associated with peripheral neuropathy (59). Today postoperative EBRT for RPS has largely been abandoned due to the potential

toxicity to structures that fill the tumor bed after resection and the difficulty in defining the tumor bed accurately once the tumor has been resected.

IORT with electron beam combined with preoperative EBRT may also reduce local recurrence compared to EBRT alone (183), but is not widely used due to its limited availability. IORT with catheter brachytherapy has been evaluated with catheters placed against the operative bed at the end of the procedure for postoperative loading with iridium (^{192}Ir). In the University of Toronto/Princess Margaret Hospital experience with this approach, two patients with RPS died after duodenal perforations (184). Therefore, this practice has largely been abandoned.

Preoperative EBRT offers several potential advantages over other radiation approaches. The tumor displaces critical structures such as small bowel out of the way. Furthermore, the gross tumor volume may be more precisely defined. A randomized trial evaluating the efficacy of preoperative EBRT was launched by the American College of Surgeons Oncology Group, but was halted due to poor accrual. A similar phase III trial is currently underway under the auspices of EORTC. There are no established guidelines for preoperative EBRT, however consensus recommendations were recently developed (185). The recommended dose is 5040 cGy, delivered in 180 cGy fractions. Intensity modulated radiation therapy is the preferred treatment technique. Dose to the liver and contralateral kidney should be minimized, and doses to other organs are being defined. The clinical target volume for radiation delivery should be expanded by 1.5 cm beyond the gross tumor volume in general, but this can be edited depending on the specific tissue or organ within the clinical target volume.

Proton beam radiation therapy employs protons instead of photons. This technique offers the advantage of low entrance dose and minimal exit dose, which is particularly critical around structures such as the spinal cord. Like IORT with electron beam, proton beam radiation therapy is only available at a limited number of institutions.

In summary, the benefit of radiation therapy in retroperitoneal sarcoma has not been established. Preoperative EBRT offers the most promising approach and is currently under evaluation in an ongoing phase III trial. Postoperative EBRT and IORT with catheter brachytherapy are best avoided. IORT with electron beam and proton beam radiation therapy may be considered where available, though their superiority to other approaches has not been demonstrated.

Chemotherapy

The data for chemotherapy for primary RPS is quite limited. Meric and colleagues evaluated doxorubicin- or ifosfamide-based neoadjuvant regimens and found that in the subset of patients with RPS, none demonstrated a response meaningful enough to permit organ salvage (186). Donahue and colleagues found that patients receiving chemotherapy had no better disease-specific survival than predicted by the nomogram, but in the subset of patient with a pathologic response, survival was improved (187).

4.5 Gastrointestinal Stromal Tumor (GIST)

Initial Workup

The majority of patients with GIST are symptomatic when diagnosed, and 50% have metastatic disease at presentation (188). The two most common sites of origin are stomach (60%) and small intestine (30%)(189).

Contrast enhanced CT of the abdomen and pelvis is the preferred initial imaging study (Figure 25). Since GISTs rarely metastasize to lungs (usually in later stages of disease), chest imaging is not routinely required. MRIs may offer anatomic detail for primary rectal GIST or metastases to the liver. While much has been written about the utility of PET in evaluating response to TKI therapy, PET scans are not routinely needed for initial or follow-up imaging. However, PET scans may help distinguish ambiguous findings seen on CT or help evaluate treatment response in specific circumstances.

Esophageal, gastric, proximal small bowel, and colorectal primary GISTs may be biopsied endoscopically (134). Since tumors are usually submucosal, endoscopic mucosal biopsies have much lower yield than endoscopic ultrasound (EUS)-guided fine-needle aspiration. Liver metastases may be biopsied percutaneously. However, biopsy is not necessary for all primary tumors, especially for patients undergoing surgery upfront.

Institutions are increasingly offering mutation testing. While not absolutely necessary for initial treatment, knowing the mutation status may impact selection of tyrosine kinase inhibitor (TKI) therapy in the adjuvant setting for primary disease or for metastatic disease.

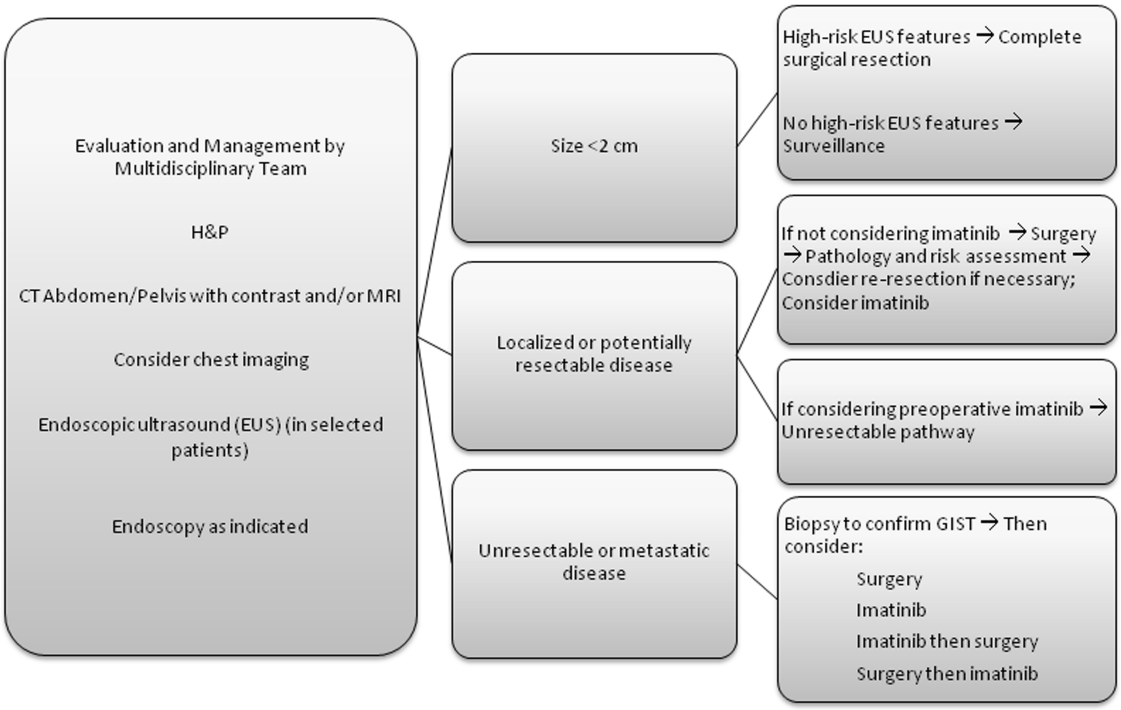


Figure 25: Overview of NCCN guidelines for management of gastrointestinal stromal tumors (GIST).

Staging and Survival Prediction

GIST is the most common mesenchymal tumor in the GI tract. The designation of GIST refers to a specific tumor type that is generally immunohistochemically KIT-positive and is driven by KIT or PDGFRA activating mutations. While there are differences in behavior between GISTs with different types of KIT and PDGFRA mutations, mutation status is not currently factored into stage due to limitations in the universal application or availability of these studies. The 7th edition AJCC manual was the first edition to propose a staging system for GIST, by offering statistical probabilities of metastatic development, based on tumor size, tumor site of origin, and mitotic rate, the most important and widely studied prognostic parameters in GIST (Table 3)(190, 191). As GISTs encompass a broad biologic continuum, generalizations

regarding GIST survival are not possible. Survival curves generated prior to 2001 are complicated by inaccuracy in GIST diagnosis, and are mostly based on single-institution studies. Those afterwards are confounded by the use of adjuvant therapy. Recurrence-free survival of localized, primary GIST after complete surgical resection may be calculated using a nomogram including the same prognostic factors (Figure 26)(192).

Surgery

Despite advances in systemic therapy, surgery remains the only potentially curative therapy for GIST. Management of tumors less than 2 cm in size is controversial. NCCN guidelines recommend that such small GISTs without high-risk EUS features may be observed with serial endoscopic surveillance. GISTs 2 cm in size or greater should be resected. The goal of surgery is a macroscopically complete resection with negative microscopic margins. Resection for GIST, unlike for other sarcomas, does not require a wide margin, but simply a negative margin. GISTs, in adults, rarely recur locally at the primary site, which is different than adenocarcinomas or other sarcomas. In fact, data from a prospective study of adjuvant use of the TKI imatinib mesylate demonstrated that patients undergoing a margin-positive (R1) resection did not have a higher risk of recurrence than those undergoing a margin-negative (R0) resection, irrespective of adjuvant imatinib use (193).

While wedge or segmental resections of the involved viscera is preferred, tumors that are large or sited in a challenging location may require more extensive resections (see section below on neoadjuvant therapy). Laparoscopic resections may be considered.

Surgery may be considered for metastatic disease. At present, patients with metastatic disease who are responding to standard TKI therapy or have focal progression on TKI therapy may be considered on case-by-case basis for cytoreductive surgery. Patients who have generalized or multifocal progression on TKI should not be considered for surgery routinely unless symptomatic, given poor outcomes (194-196). Most of the data come from single-institution studies for patients on first-line TKI imatinib (194-199); only one study has reported surgery for patients with metastatic GIST on second-line TKI sunitinib malate (200). Recent data suggest that when surgery is considered, the goal of surgery should be a macroscopically complete resection, as patients with R2 resections have worse outcome (201). After surgery, all patients should resume TKI therapy. Surgery is not an alternative to TKI therapy, and those that fail to resume drug therapy will recur quickly. It remains unclear whether surgery plus TKI therapy adds benefit in terms of recurrence-free survival or disease-specific survival over TKI therapy alone (202).

Radiation

GIST is generally a radioresistant tumor, although radiation therapy may be used for palliation on an individual basis.

Chemotherapy

The identification of gain-of-function mutations in the KIT proto-oncogene in patients with GIST provided a potential treatment target. Subsequent development of effective, orally available TKIs revolutionized the management of this malignancy.

Table 3: AJCC staging of gastrointestinal stromal tumors (GIST)*				
Primary Tumor (T)				
TX	Primary tumor cannot be assessed			
T0	No evidence of primary tumor			
T1	Tumor ≤ 2 cm in greatest dimension			
T2	Tumor >2 cm but ≤ 5 cm in dimension			
T3	Tumor >5 cm but ≤ 10 cm in dimension			
T4	Tumor > 10 cm in greatest dimension			
Regional Lymph Nodes (N)				
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph node metastasis			
N1	Regional lymph node metastasis			
Distant Metastasis (M)				
M0	No distant metastasis			
M1	Distant metastasis			
Anatomic Stage/Prognostic Groups				
Gastric GIST**				
Group	T	N	M	Mitotic Rate
Stage IA	T1 or T2	N0	M0	Low
Stage IB	T3	N0	M0	Low
Stage II	T1	N0	M0	High
	T2	N0	M0	High
	T4	N0	M0	Low
Stage IIIA	T3	N0	M0	High
Stage IIIB	T4	N0	M0	High
Stage IV	Any T	N1	M0	Any rate
		Any		
	Any T	N	M1	Any rate
Small Intestinal GIST***				
Group	T	N	M	Mitotic Rate
Stage I	T1 or T2	N0	M0	Low
Stage II	T3	N0	M0	Low
Stage IIIA	T1	N0	M0	High
	T4	N0	M0	Low
Stage IIIB	T2	N0	M0	High
	T3	N0	M0	High
	T4	N0	M0	High
Stage IV	Any T	N1	M0	Any rate
		Any		
	Any T	N	M1	Any rate

*Reprinted with permission from AJCC 7th Ed.

** Note: Also to be used for omentum

*** Note: Also to be used for esophagus, colorectal, mesentery, and peritoneum

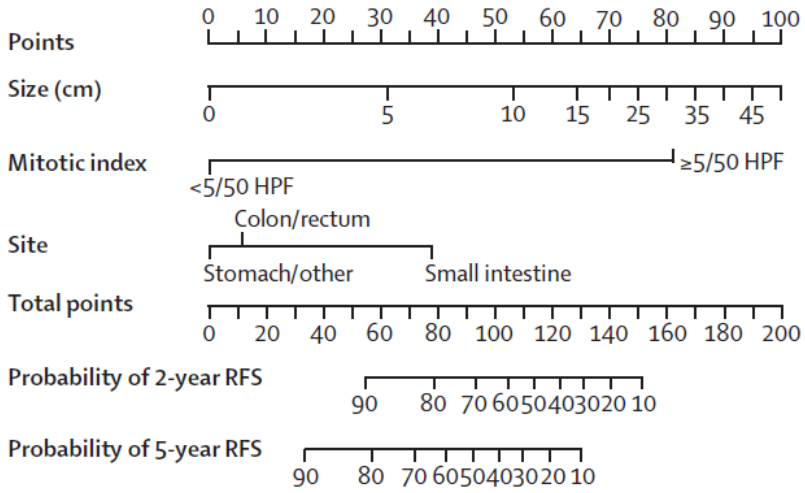


Figure 26: Nomogram to predict the probabilities of 2-year and 5-year recurrence-free survival of GIST (Gold et al 2009). HPF, high-powered fields.

Imatinib mesylate, a tyrosine kinase inhibitor (TKI) which targets KIT, PDGFRA, and bcr-abl has shown to dramatically improve progression-free and overall survival outcomes in a series of trials in the US and Europe (203-206). Over 80% of patients with advanced or metastatic GIST treated with imatinib respond (205, 206). Imatinib is now considered first-line therapy for advanced stage or metastatic GIST.

Sunitinib mesylate is a multi-targeted TKI approved by the US Food and Drug Administration as second-line therapy for patients resistant to or intolerant of imatinib. Regorafenib, another multi-targeted TKI was recently approved by the FDA as third-line therapy for patients progressing on imatinib and sunitinb.

The efficacy of imatinib in patients with metastatic disease prompted investigators to evaluate imatinib as an adjuvant and neoadjuvant agent in patients with primary GIST. Phase III studies have compared placebo to 1 year (ACOSOG Z9001) and 2 years (EORTC 62023) of adjuvant imatinib and 1 year to 3 years (SSG XVIII) of adjuvant imatinib (207-209). Together, these adjuvant imatinib improved recurrence-

free survival, essentially delaying recurrence, without necessarily curing patients. Three years of adjuvant imatinib did improve overall survival compared to 1 year. The current standard of care is 3 years of adjuvant imatinib following resection (134).

Several single-institution and multi-institution studies have evaluated neoadjuvant imatinib for primary GIST (210, 211). Indications for neoadjuvant therapy include downstaging tumor size to minimize the scope of an operation (segmental duodenal resection instead of pancreaticoduodenectomy for duodenal GIST, transanal resection instead of abdominoperineal resection for rectal GIST) or approach to an operation (minimally invasive gastrectomy or esophagogastrectomy instead of open procedures).

These studies have demonstrated that neoadjuvant therapy is safe. The ideal length of therapy is uncertain, but recent data suggest that maximal radiographic responses may not be observed until approximately 28 weeks (212). Current practice is to continue neoadjuvant treatment for approximately 6 months or until a radiographic response plateaus.

4.6 Future Directions

The rapid evolution of understanding in cancer biology and the availability of biologic factors that predict cancer outcome and response to treatment in GIST foreshadow the potential opportunity to identify similar factors in other soft tissue sarcomas. The AJCC 6th edition staging manual introduced judicious consideration of nonanatomic prognostic factors as modifiers to the anatomic T, N, and M groupings. The current AJCC 7th edition separates staging for GIST from other sarcomas, and has added mitotic rate as a nonanatomic prognostic modifier. There are no

recommendations for the inclusion of nonanatomic prognostic factors for other soft issue sarcomas within the current AJCC 7th edition.

Given the successful and unique management strategy adopted for GIST, further studies are underway attempting to identify other soft tissue sarcomas that may have unique molecular or biologic signatures that may be amenable to targeted systemic therapies.

CHAPTER 5: OPERATIVE MANAGEMENT OF A MALIGNANT PHEOCHROMOCYTOMA LONG BONE METASTASIS: CASE REPORT AND REVIEW OF PERIOPERATIVE CONSDIERATIONS

5.1 Introduction

Pheochromocytomas (PCCs) are neuroendocrine tumors with an incidence of 2-10 cases per million persons per year (213, 214). PCCs most commonly arise in the adrenal medulla, while approximately 20% arise from extra-adrenal chromaffin tissue (termed paragangliomas). The majority of cases are sporadic, however, up to 30% of cases may be linked to a hereditary syndrome (215) including von Hippel Lindau disease (VHL), multiple endocrine neoplasia type 2 (MEN2), or neurofibromatosis type 1 (NF1). Due to production of catecholamines by these tumors, hypertension is the most common clinical feature at presentation.

Malignant PCCs are extremely rare, developing in approximately 13% of patients diagnosed with PCC (216). Malignancy is not reliably distinguishable by histologic examination of primary tumors. Rather, malignancy is defined by the presence of metastasis in anatomic regions where chromaffin tissue is normally absent. The most common sites of metastases are the bones, lymph nodes, liver, and lungs (217-219). The 5-year overall survival rate of patients with malignant PCC is approximately 40%, but patients with isolated bone metastases tend to have better prognosis than those with liver or lung metastases (217).

Bone metastases (BMs) account for 35-71% of distant metastases of malignant pheochromocytoma (217-219). In the largest case series of 91 patients with BMs, 38% of these BMs were synchronous with the primary tumor, while 63% were metachronous (217). In this study, 77% of BMs were widespread. The most common locations were the spine (81%), sacrum and pelvis (67%), proximal and distal long bones (49%), and skull (21%). While there are many reports of operative management of malignant PCC BMs in the spine (220-227), there are few reports of long bone metastasis requiring operative management (228-230). Here we report the operative management of a catecholamine-producing malignant PCC metastasis to the proximal femur and provide a useful clinical algorithm for orthopaedic surgeons who infrequently manage this type of tumor.

5.2 Case Report

The patient provided consent for the data concerning his case to be submitted for publication. This Caucasian male initially presented for a routine history and physical examination at the age of 60, during which his primary care provider noted a large mass on abdominal exam. He was 6'1", weighed 190 pounds, and had a body mass index of 25. His comorbidities included insulin-dependent diabetes mellitus, hypertension, hypercholesterolemia, chronic kidney disease stage 2, hypothyroidism, and peripheral arterial disease. His family history was significant for breast and lung cancer in his mother. He drank 1-2 alcoholic beverages per day and was a nonsmoker. Workup of the patient's abdominal mass revealed a 10 x 10 x 14 cm retroperitoneal tumor, and core biopsy revealed a malignant neoplasm. He underwent open resection of this large mass, however, intraoperatively he was found to also have a smaller left adrenal tumor 1.5 cm

in greatest dimension. Pheochromocytoma had not been suspected in this patient, and intraoperatively he sustained a hypertensive crisis with systolic blood pressure >300 mmHg. Pathology revealed that both masses were positive for synaptophysin, chromogranin, S-100 and inhibin. The patient was diagnosed with primary adrenal pheochromocytoma, with regional retroperitoneal spread versus a synchronous primary paraganglioma.

The patient underwent routine follow-up and periodic labwork including urine metanephrines, vanillylmandelic acid (VMA), chromogranin A, and 5-hydroxyindoleacetic acid (5-HIAA). 24 months after his primary resection, he developed elevated urine chromogranin A and metanephrines compared to his baseline labs. Imaging revealed metastatic disease including pulmonary and hepatic lesions, and a right proximal femur metastasis. He underwent chemotherapy, first with a course of cyclophosphamide, vincristine, and dacarbazine for 3 months with no improvement. He then underwent four months therapy in a clinical trial with etoposide, cisplatin, and belinostat resulting in stable disease.

At the age of 63, 34 months following his primary surgery, the patient presented with right-sided groin pain with weight bearing. Physical examination was significant for pain with resisted straight leg raising on the right. Metaiodobenzylguanidine (MIBG) scan and positron emission tomography (PET) scan revealed worsened metastatic disease with hypermetabolic lesions in the lungs, liver, ribs, left scapula, left ilium, sacrum, multiple thoracic and lumbar vertebrae, and right subtrochanteric femur. MRI imaging revealed an expansile radiolucent lesion in the right subtrochanteric femur with thinned cortices (Figure 27). The patient underwent 10 cycles of palliative

external beam radiation therapy with 30 Gy to his femur, with no alleviation of his pain. Given his risk for impending pathologic femur fracture, the patient was offered prophylactic intramedullary nailing of the right femur. The benefits and risks of this surgery, including the risk for hypertensive crisis, were discussed with the patient and he consented to the procedure.

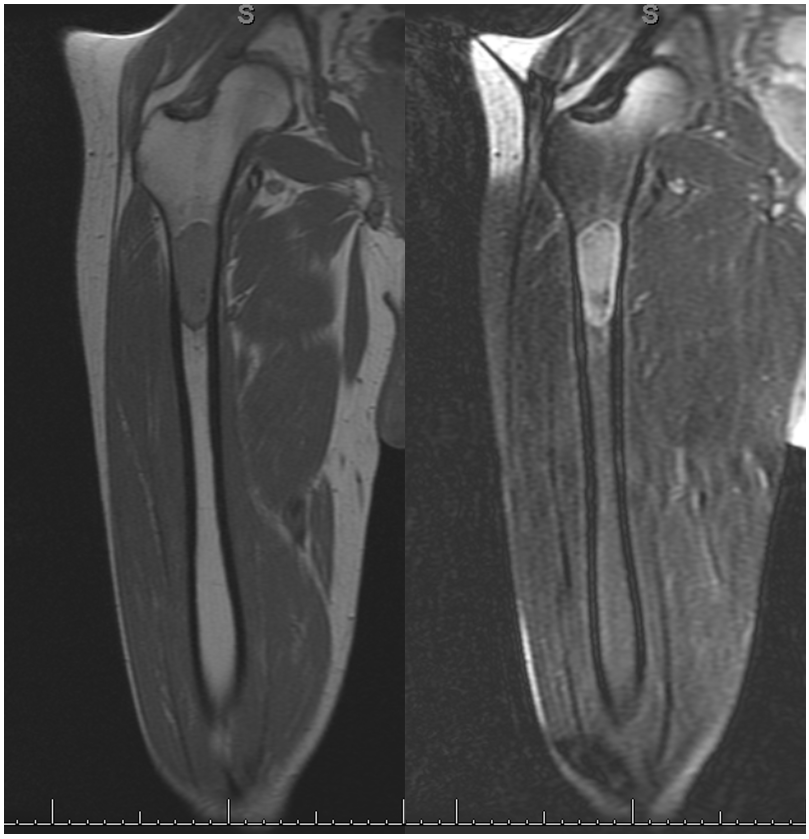


Figure 27: Magnetic resonance imaging (MRI) of right femur showing a subtrochanteric lesion with thinning of the cortices. T1 weighted image (left) and proton density weighted image (right).

The patient underwent preoperative clearance with anesthesia, which included basic labs (BMP and CBC), as well as electrocardiogram. The patient had been on chronic alpha-adrenergic blockade with phenoxybenzamine, and therefore no new alpha

blockade was needed. General anesthesia was planned with central venous and arterial blood pressure monitoring, followed by postoperative overnight monitoring in the intensive care unit.

The patient underwent prophylactic intramedullary nailing of the right subtrochanteric femur utilizing a lateral entry cephalomedullary nail with two proximal locking bolts and two distal screws (Figure 28). After initial entry reaming of the proximal peritrochanteric femur, a chest tube was utilized to aspirate the intramedullary canal contents prior to subtrochanteric and diaphyseal reaming (231). Given the high risk for hypertensive crisis, during the procedure the surgeon and the anesthesiologist communicated regularly regarding the degree to which the tumor was being manipulated. The patient's blood pressure was appropriately controlled throughout the 1 hour and 27 minute procedure. Post-operatively the patient was extubated and monitored overnight in the intensive care unit as planned. The hospitalist service was consulted and recommended restarting the patient's phenoxybenzamine on post-operative day 0. The patient was transferred to the regular hospital floor on post-operative day 1. The patient experienced symptomatic orthostatic hypotension when attempting physical therapy, necessitating discontinuation of the patient's phenoxybenzamine. The patient's orthostatic hypotension resolved slowly over the next few days, and the patient was discharged on post-operative day 5.

Pathology confirmed metastatic pheochromocytoma of the right femur (Figure 29), with positive stains for chromogranin, synaptophysin, and S-100. At six month post-operative follow-up visit, the patient had an excellent surgical outcome and had achieved radiographic union of his fracture site. He had returned to work and was

ambulating at home and in the office home and office without assistive devices. He had completed a clinical trial for MIBG radioisotope therapy and had stable clinical disease.



Figure 28: Post-operative radiograph of the right femur status post intramedullary nail placement for impending pathologic fracture.

5.3 Discussion

Malignant pheochromocytoma bone metastases are exceedingly rare; however, they do infrequently require orthopaedic evaluation and management. Bone lesions which arise in a patient with a history of pheochromocytoma should be approached similarly to other suspected bone metastases (Figure 30). There are no effective treatment options for malignant pheochromocytoma, however a survival benefit has

been shown amongst responders to chemotherapy with cyclophosphamide, vincristine, and dacarbazine (232, 233). Radioisotope treatment of metastases with MIBG uptake has also shown a modest survival benefit (234, 235), however it tends to be less effective for bone metastases (235). External beam radiation therapy or radiofrequency ablation may be considered for palliation of painful lesions, however there is limited evidence to support these approaches (236, 237).

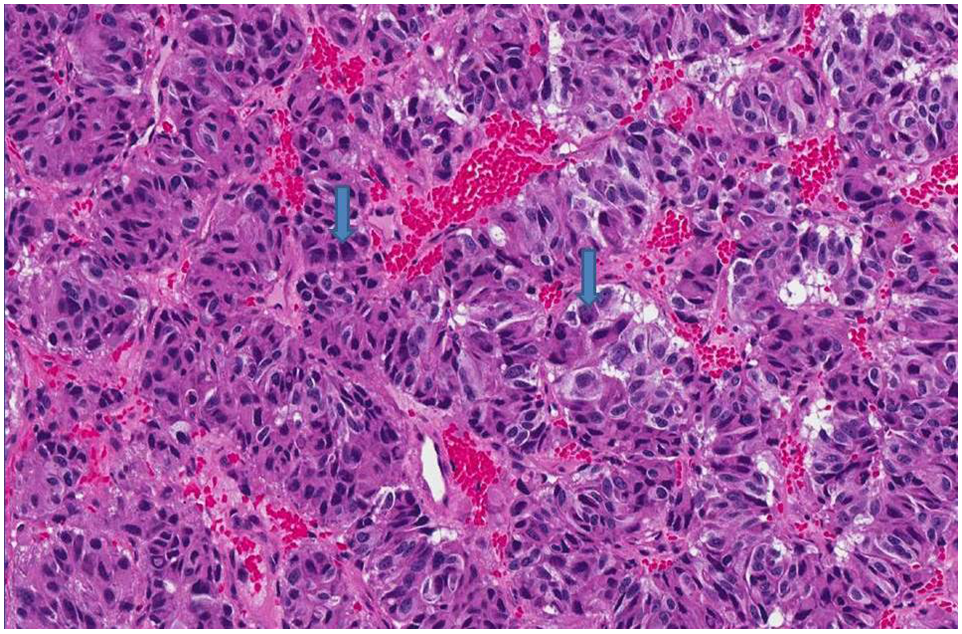


Figure 29: High power photomicrograph of pheochromocytoma bone metastasis showing a relatively uniform population of polygonal cells with eosinophilic cytoplasm. Fibrovascular stroma outline nests of tumor cells, and focal nuclear atypia is present (arrows) (H&E 200x).

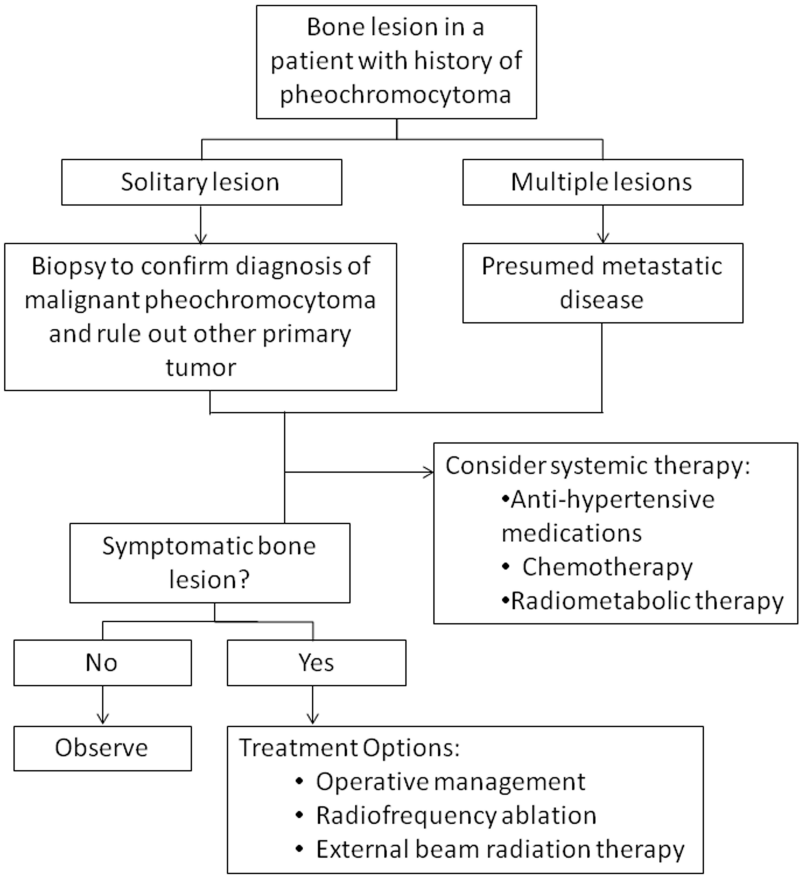


Figure 30: Clinical algorithm for management of pheochromocytoma bone metastases. Adapted from NCCN guidelines for metastatic disease.

Surgical palliation of bone metastases may be considered with in patients with refractory hypertension, refractory pain, impending or established pathologic fracture (238), neurologic compromise, or other pertinent skeletally-related event (Table 4). Preoperative consultation should include a thorough discussion with the patient regarding the goals for the procedure and the risks for perioperative morbidity and mortality, with particular attention to the development and prevention of malignant hypertension. Other important perioperative considerations in management are outlined in Table 4 (239-241).

Our case describes a malignant pheochromocytoma metastasis to the proximal femur requiring operative treatment. These lesions may be safely managed surgically, with careful consideration of the unique features of pheochromocytoma and appropriate interdisciplinary care.

Table 4: Perioperative considerations in management of pheochromocytoma bone metastases
Operative Indications
Refractory hypertension
Refractory pain
Pathologic fracture
Impending pathologic fracture
Neurologic compromise
Operative Goals
Palliation of pain
Functional improvement
Increased independence
Prevention of pathologic fracture
Pre-operative Considerations
Thorough history and physical exam
CBC, BMP, EKG, anesthesia evaluation
Alpha adrenergic blockade 1-2 weeks
Intravascular volume preparation (if hypovolemic)
Avoidance of foods and medications that increase catecholamine synthesis
Consider pre-operative radiation therapy or embolization
Operative considerations
Minimize tumor manipulation
Send frozen pathology sample
Obtain adequate blood pressure monitoring and vascular access (arterial line, central line, large bore peripheral IV)
Monitor for hypertensive crisis, reflex tachycardia, and other arrhythmias
Post-operative considerations
24-hour close monitoring for hypotension, hypoglycemia, and arrhythmias
Consider dextrose in IV fluids to prevent hypoglycemia

CHAPTER 6: INJURY TYPE AND EMERGENCY DEPARTMENT MANAGEMENT OF ORTHOPAEDIC PATIENTS INFLUENCES FOLLOW-UP RATES

6.1 Abstract

Background: Orthopaedic clinic follow-up is required to ensure optimal management and outcome for many patients presenting to the Emergency Department (ED) with orthopaedic injuries. While several studies have shown that demographic variables influence emergency patient follow-up, the objective of this study was to examine the orthopaedic-related factors associated with failure to return, so-called "no-show," after ED visit.

Methods: A chart review was conducted at a large academic public hospital. Four hundred sixty four (464) consecutive adult patients receiving an orthopedic consult in the ED with subsequent referral to orthopedic clinic from January to June 2011 were included. Data regarding injury type and management were analyzed for association with no-show using Chi-squared and Mann Whitney univariate tests. Variables with $p<0.25$ were included in a multivariate stepwise forward logistic regression analysis.

Results: The overall no-show rate was 26%. Logistic regression modeling revealed significant differences based on cause of injury (odds ratio [OR] 7.51, 95% confidence interval [CI] 2.27-25.1) with assault victims having the highest no-show rates. Anatomic region of injury was significant (OR 6.61, CI 1.45-30.5) with patients with spinal injuries having the highest no-show rates. Follow-up rates were provider-

specific among orthopaedic residents (OR 10.8, CI 4.11-31.1), and this was not related to level of training (p=0.25). Type of bracing applied influenced the no-show rate (OR 2.46, CI 1.58-3.96), and the easier it was to remove the brace (splint), the worse the follow up (p=0.0001). Several demographic variables were also predictive of nonattendance, including morbid obesity (OR 15.0, CI 4.83 - 51.6) and current smoking (OR 5.56, CI 2.19-15.4).

Conclusions: This study supports previous evidence of high no-show rates in emergency patients with scheduled orthopaedic follow-up. Furthermore, the data highlights distinct orthopaedic- related factors associated with nonattendance. These findings are useful in identifying patients at high risk for no-show to scheduled orthopaedic follow-up appointments and may influence disposition and management decisions in these patients.

6.2 Introduction

Orthopaedic clinic follow-up is required to ensure optimal management and outcome for many patients presenting to the Emergency Department (ED) with orthopaedic injuries. No-show rates after ED visit have ranged from 7% for follow-up in a managed care system (242) to 72% for primary care follow-up in an academic system (243).

Several previous studies have found associations between no-show and sociodemographic variables, and systems factors have also been implicated (242-246). No-show rates can vary significantly by specialty (243, 246, 247), however, few studies have investigated orthopaedic-specific factors related to no-show after ED visit (243, 248, 249).

The purpose of the present study was to determine the patient, hospital system, management, and orthopaedic-related factors associated with no-show rates. Identifying patients at risk of no-show may allow providers to implement alternative strategies designed to improve follow up, specifically targeted to this population.

6.3 Methods

Study Setting and Population

This study was approved with a waiver of informed consent by the institutional review boards (IRBs) of the county hospital health system and the affiliated academic institution (Baylor College of Medicine). A chart review was conducted at a large public hospital with greater than 100,000 ED visits annually and which serves as the primary training site for both orthopaedic and emergency medicine residency programs. This hospital is a 'staff model' managed health system, which serves a large proportion of low-income, uninsured, and minority individuals. The hospital is part of a county hospital system which offers within-system healthcare coverage for in-county residents who financially qualify. The primary responders for orthopaedic consultations are first and second year orthopaedic surgery residents on a rotating schedule.

With regards to compliance with outpatient appointments, there are no specific incentives or reminders built into the system. Approximately one third of patients are given an outpatient appointment at the time of ED discharge by the ED cashier. The scheduler is not given permission to overbook patients. Therefore, if the orthopaedic clinic schedule is already full for the desired day, the patient leaves the ED without an appointment. They are subsequently contacted directly by the orthopaedic clinic to confirm the appointment (by phone if the appointment is in the upcoming week, or by

mail if it is greater than one week away). If a patient is unwilling to confirm an appointment time at discharge, they may call a scheduling hotline to make the appointment after the visit rather than waiting to be contacted by the clinic.

The orthopaedic service maintains daily records of all patient consults received. The electronic medical record (EMR) of all 1,337 consecutive patients receiving an orthopaedic consult from the ED between 1 January and 30 June 2011 was reviewed by one of three authors to determine eligibility. Exclusion criteria were age less than 18 years, patients admitted to the hospital for any reason, those who left against medical advice prior to discharge, or patients for whom no orthopaedic clinic follow-up was recommended (Figure 31). Four hundred sixty four (464) patients met inclusion criteria and were included in this study.

Data Collection

Patient demographic factors were recorded including age; sex; race; primary language; United States residency status; marital status; body mass index (BMI); presence or absence of tobacco use, alcohol abuse and drug use; psychiatric comorbidity; number of primary care provider (PCP) visits in the past year; number of ED visits in the past year; insurance status; whether the patient had financial assistance through the hospital system; whether the patient was visiting the hospital system for the first time or was an established patient; whether the patient was in police custody during their ED visit; and whether the patient sustained the injury as the victim of a crime. The zip code of the patient's home address was recorded and matched to the median household income bracket for each zip code based on the Internal Revenue Service (IRS) 2008 individual income return data (250). The patient's home address was also

entered into Google Maps to calculate the driving distance from the patient's home to the hospital (251).

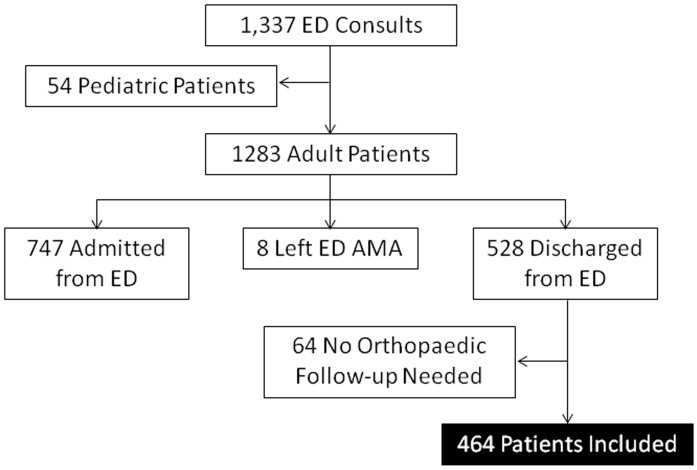


Figure 31: Patient inclusion algorithm.

Orthopaedic factors recorded included whether the problem was already being managed by the hospital's orthopaedic department; the mechanism of injury (blunt versus penetrating trauma); the cause of injury (fall, assault, motor vehicle accident, machinery and power tools, or other); the anatomic region of the orthopedic problem; how long prior to the ED visit symptoms began; the training level of the orthopaedic staff caring for the patient; the specific orthopaedic provider caring for the patient; any procedure performed in the ED (for example, fracture reduction); whether moderate (conscious) sedation was used during an ED procedure; the type of splint placed in the ED; whether antibiotics or narcotics were prescribed upon ED discharge; and the type of follow-up recommended (scheduled surgery versus clinic appointment).

ED factors recorded were whether the patient had been referred to the ED by an outside provider; the length of stay in the ED; the number of handoffs between ED

providers; the training level of the ED provider; when the follow-up appointment was scheduled for the patient (prior to ED discharge versus later); and whether the patient returned to the ED prior to their scheduled follow-up appointment.

Statistical Analysis

Descriptive statistics were calculated for all variables collected. Nominal variables were categorized as binary variables to ensure adequate numbers in each comparison group. Categorical variables were analyzed with Chi-squared Contingency Test, and continuous variables were analyzed with Mann-Whitney Test for association with the primary outcome of no-show to the orthopaedic clinic.

Forward stepwise multivariate logistic regression was performed with p_{ENTER} <0.25 and p_{REMOVE}>0.30. Age and sex were included in the model *a priori* given their clinical relevance and known impact on follow-up in previous studies. Variables with p >0.10 were removed if they did not have an impact on the overall model, as assessed by Bayesian information criterion (BIC). Several alternative models were constructed using different categorizations of nominal variables without difference in overall significance of the model. Importance of clinically feasible interactions was considered. Discrimination of the final model was assessed using the area under the receiver operating characteristic (ROC) curve (252) to evaluate how well the model distinguished patients who followed-up versus those who did not (Figure 32). Associations were estimated on the basis of odds ratios (ORs) and 95% confidence intervals (CIs). p-value <0.05 (two-tailed) was considered to be statistically significant.

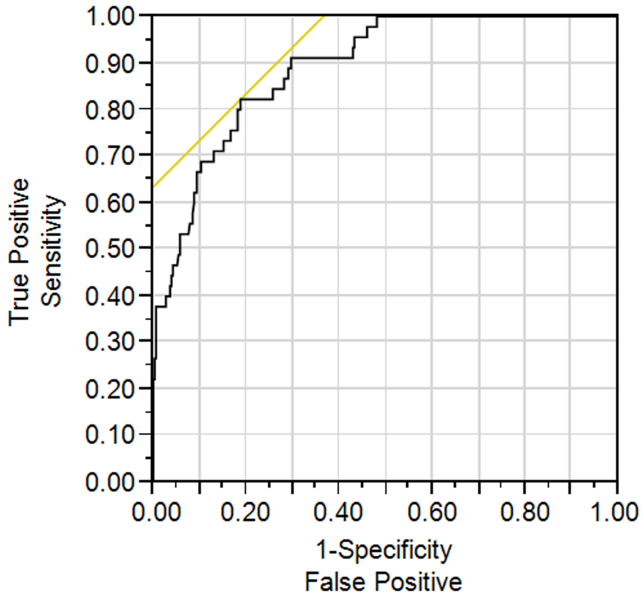


Figure 32: Receiver Operating Characteristic (ROC) Curve for prediction of no show. The ROC curve was generated by plotting sensitivity against 1 minus the specificity for the logistic regression model. The area under the ROC curve was 0.89.

6.4 Results

The overall no-show rate was 26.1% (121/464). Table 5 presents all variables evaluated, separating those factors that did or did not have significant effects on no-show rate based on $p<0.05$ on univariate analysis. Table 6 shows the detailed analysis of those variables that were significant on univariate analysis. Several orthopaedic factors were significantly associated with no-show (Figure 33). Factors that remained significantly predictive of no-show on multivariate logistic regression analysis are shown in Table 7.

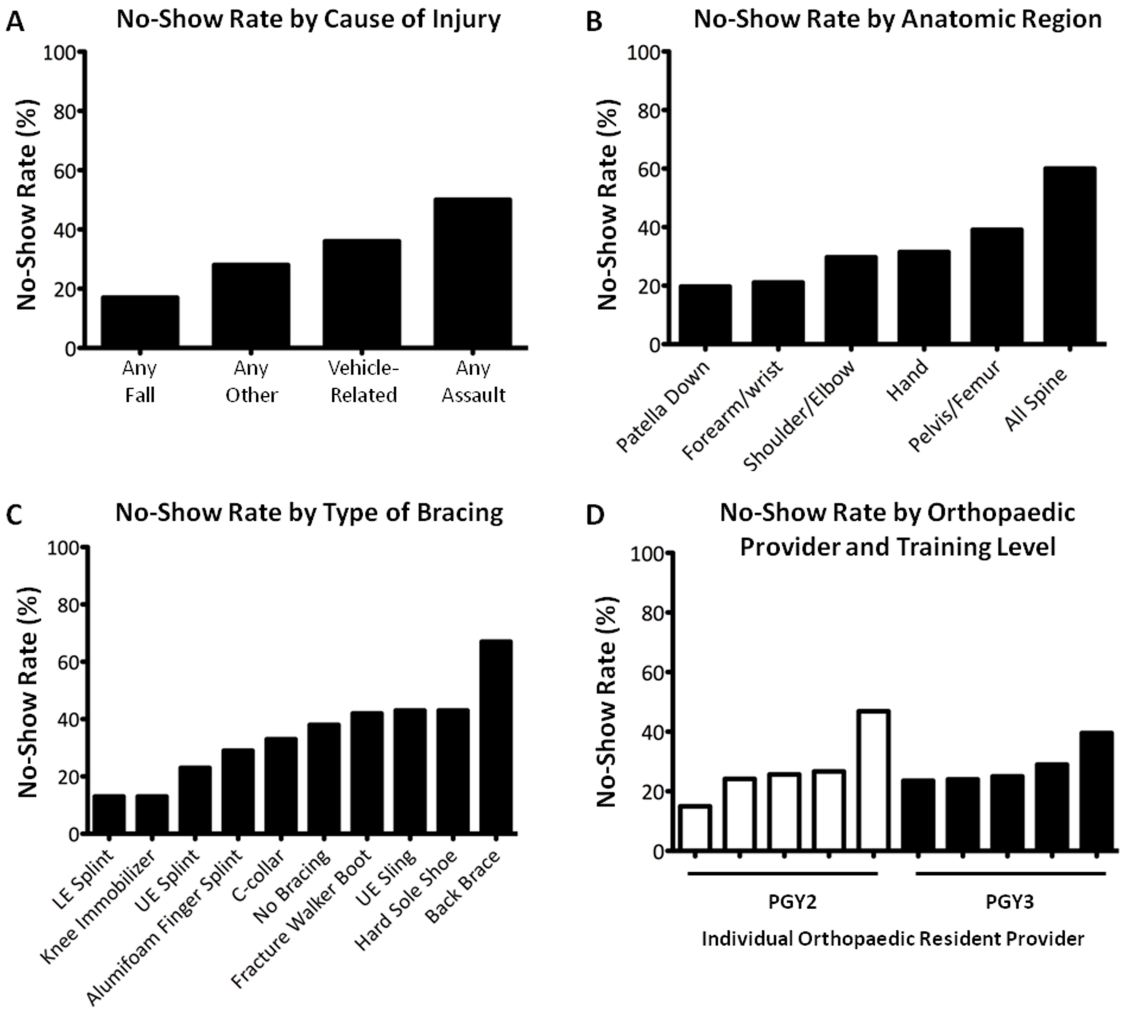


Figure 33: Rates of no-show for orthopaedic variables significant on logistic regression modeling. LE = lower extremity. UE = upper extremity. Back Brace = any thoracic or lumbar bracing. Patella down = all regions from the patella to toes including leg and foot. PGY = Post-graduate year in residency training.

Logistic regression modeling revealed significant differences based on cause of injury (odds ratio [OR] 7.51, 95% confidence interval [CI] 2.27-25.1) with assault victims having the highest no-show rates. Anatomic region of injury was significant (OR 6.61, CI 1.45-30.5) with patients with spinal injuries not requiring admission having the highest no-show rates. Follow-up rates were provider-specific among orthopaedic residents (OR 10.8, CI 4.11-31.1), and this was not related to level of training (p=0.25). Type of bracing applied influenced the no-show rate (OR 2.46, CI

1.58-3.96), and the easier it was to remove the brace (splint), the worse the follow up (p=0.0001). Several demographic variables were also predictive of nonattendance, including morbid obesity (OR 15.0, CI 4.83 - 51.6) and current smoking (OR 5.56, CI 2.19-15.4).

Table 5: Factors analyzed in univariate analysis of nonattendance at follow-up visit *(listed in order of decreasing significance)*

Variables with significant influence (p<0.05)	Variables with non-significant influence (p≥0.05)
Tobacco use	Age
Insured in our health system	Patient is a crime victim
Cause of injury	Annual income
Type of immobilization applied	Drug use history
Training level of ED provider	Alcohol abuse history
Individual orthopedic provider	Antibiotics prescribed
Type of procedure performed in ED*	Orthopaedic provider PGY‡ training level
Type of follow up – surgery vs. clinic	Discharged with narcotic prescription
Body mass index (BMI)	Mechanism of scheduling appointment
Number of PCP† visits prior year	Length of ED stay
Anatomical site of injury	Psychiatric comorbidity
Sex	Marital Status
Primary Language	Mechanism of injury: blunt or penetrating
Race	Patient in police custody
How patient was referred to ED	Moderate (conscious) sedation in ED
Patient new to our orthopedists	Return to ED prior to scheduled appointment
Number of ED Provider handoffs	Number of ED visits per year
Distance from patient's home to hospital	* ED - Emergency Department
Insurance status	† PCP - Primary Care Provider
New to our health system	‡ PGY - Post-graduate year. This variable and those below were not included in the logistic regression given p>0.25.
Residency status	

Table 6: Univariate analysis of variables associated with nonattendance at follow-up visit. Only variables with p <0.05 included.

DEMOGRAPHIC FACTORS				
Characteristic	Total Number (%)	Follow-up Rate (%)	No-Show Rate (%)	p Value*
Sex				0.0024
Male	284 (61)	69	31	
Female	180 (39)	82	18	
Race				0.0117
Asian/Pacific Islander	12 (3)	67	33	
Black/African American	115 (25)	73	27	
Hispanic/Latino	241 (52)	79	21	
White/Caucasian	95 (21)	62	38	
Missing	1			
Primary Language				0.0073
English	306 (67)	70	30	
Other	154 (33)	82	18	
Missing	4			
Residency Status				0.0432*
U.S. Born American	280 (61)	70	30	
Non U.S. Born	176 (39)	80	21	
Missing	8			
Body Mass Index (BMI)‡				0.0005*
Normal weight <25	129 (33)	87	13	
Overweight-Obese 25-34.9	209 (54)	91	9	
Morbidly Obese 35+	49 (13)	71	29	
Missing	77			
Distance to Hospital†				0.0244
<5 miles	40 (9)	80	20	
5 to 35 miles	395 (85)	75	25	
>35 miles	28 (6)	50	50	
Missing	1			
Tobacco Use				<0.0001*
Current smoker	176 (40)	65	35	
Non-smoker (never or former)	265 (60)	85	15	
Missing	23			
Number of Primary Care Visits in Prior Year				0.0009*
None	348 (75)	70	30	
1 to 4	90 (19)	89	11	
5 or more	26 (6)	77	23	
Insurance Status				0.0371
Private	19 (4)	53	47	
Medicaid/Medicare	51 (11)	67	33	
Uninsured	390 (84)	76	24	

Table 6 (continued)

Insured in our Health System				<0.0001*
Any level of coverage	158 (34)	90	10	
No coverage in our system	306 (66)	65	35	
New to our Health System				0.0389*
New patient	229 (49)	69	30	
Established patient	235 (51)	78	22	
ORTHOPAEDIC FACTORS				
New to Our Orthopaedic Surgeons				0.02
New to our department	438 (94)	74	26	
Established patient	26 (6)	69	31	
Cause of Injury‡				<0.0001*
Assault	40 (9)	50	50	
Fall	199 (43)	17	83	
Motor-vehicle accident	74 (16)	36	64	
Machinery/Power tool accident	21 (4)	24	76	
Other	129 (28)	29	71	
Site of Injury				0.001*
Shoulder/Elbow	64 (14)	70	30	
Forearm/Wrist	90 (19)	79	21	
Hand	89 (19)	69	31	
Spine	20 (4)	40	60	
Pelvis/Femur	23 (5)	61	39	
Patella and Below	178 (38)	80	20	
Individual Orthopaedic Provider§				0.0004*
High Risk Provider (n=2)	75 (16)	57	43	
All Other Provider (n=8)	389 (84)	77	23	
Type of Procedure Performed in Emergency Department (ED)				0.0004
Closed Reduction and/or Splinting	416 (67)	81	19	
Completion Amputation	12 (2)	83	17	
Other (Laceration Repair, Irrigation & Debridement, Arthrocentesis)	62 (10)	63	37	
No Procedure	130 (21)	65	35	
Type of Bracing Applied				<0.0001*
Plaster Splint, Alumifoam Splint or Knee Immobilizer	295 (64)	81	19	
Sling, C-Collar‖, Back Brace, Walking Boot, Toe buddy taping, No Bracing	169 (36)	60	40	

Table 6 (continued)

Type of Follow-up Recommended				0.0004
Clinic	416 (91)	72	28	
Surgery	43 (9)	95	5	
Unclear	5			
EMERGENCY DEPARTMENT FACTORS				
How Patient Was Referred to ED				0.02
Outside Provider	167 (36)	80	20	
Self-referred	297 (64)	70	30	
Number of ED Provider Handoffs†				0.0223
One or less	424 (91)	75	25	
Two or more	40 (9)	55	45	
Training Level of ED Provider Seen				<0.0001
Attending	35 (8)	83	17	
Mid-Level[‖ ¶]	203 (44)	82	18	
Resident	226 (49)	65	35	

*Remained significant on multivariate logistic regression analysis

† Individual groups presented for context, but variables analyzed as continuous data

‡ Individual groups presented for context, but variables analyzed as body mass index (BMI): ≥ 35 compared to <35 and Cause of Injury: Assault compared to All Other

§ High Risk Providers were 2 orthopaedic resident consulting providers with higher no show rates than the other 8 orthopaedic resident providers

‖ C-collar - cervical collar

¶ Mid-level providers included physician assistants and nurse practitioners

Table 7: Multivariate logistic regression analysis of variables associated with no-show at follow-up visit

Characteristic (vs. Reference)	*p* Value	Odds Ratio (Confidence Interval)
Age*	0.2003	0.19 (0.01-2.26)
Male Gender (Female)	0.4568	1.43 (0.56-3.81)
Morbid Obesity, BMI† ≥35 (BMI <35)	<0.0001	15.0 (4.83-51.6)
US Born American (All Other)	0.0266	4.80 (1.20-19.5)
Primary Language English (Not English)	0.1461	2.65 (0.71-10.0)
Annual Income ≥ $50,000 (<$50,000)*	0.2065	2.78 (0.46-12.9)
New to our Health System (Known)*	0.0073	4.77 (1.61-16.1)
Not Insured in our Health System (Any Level of Coverage in Our System)*	0.0283	3.01 (1.17-8.45)
<5 PCP‡ visits in past 12 months (≥5)	0.0343	2.12 (1.05-4.28)
Current Tobacco Use (No Current Use)	0.0005	5.56 (2.19-15.4)
Assault (Any Other Cause of Injury)	0.0009	7.51 (2.27-25.1)
Anatomic Region: Spine (Pelvis & Extremities)	0.0138	6.61 (1.45-30.5)
Other Brace or No Splint (Plaster and/or Alumifoam Splint or Knee Immobilizer)	<0.0001	2.46 (1.58-3.96)
High Risk Orthopaedic Provider (All Other Providers)	<0.0001	10.8 (4.11-31.1)
Cross Interactions:		
Age x Insurance Coverage in System	0.0127	
Income x New to Our Health System	0.0447	

* Involved in significant cross interaction
† BMI - Body mass index
‡ PCP - Primary Care Provider

There were significant cross-interactions between age and insurance coverage in the health system (p=0.0127), and between income and patients new to the health system (p=0.0447). While increasing age tended to decrease no-show in all patients, this effect was more pronounced amongst patients with insurance coverage in the system (Figure 4). Having an income greater than $50,000 U.S. dollars per year as of

2008 tended to increase no-show, and this was more dramatic amongst patients who had been seen previously at our hospital (Figure 34).

Overall, the variables associated with highest risk for no-show (OR>5), were morbid obesity, tobacco use, the individual orthopaedic provider, being injured by assault, and presenting with a spine injury.

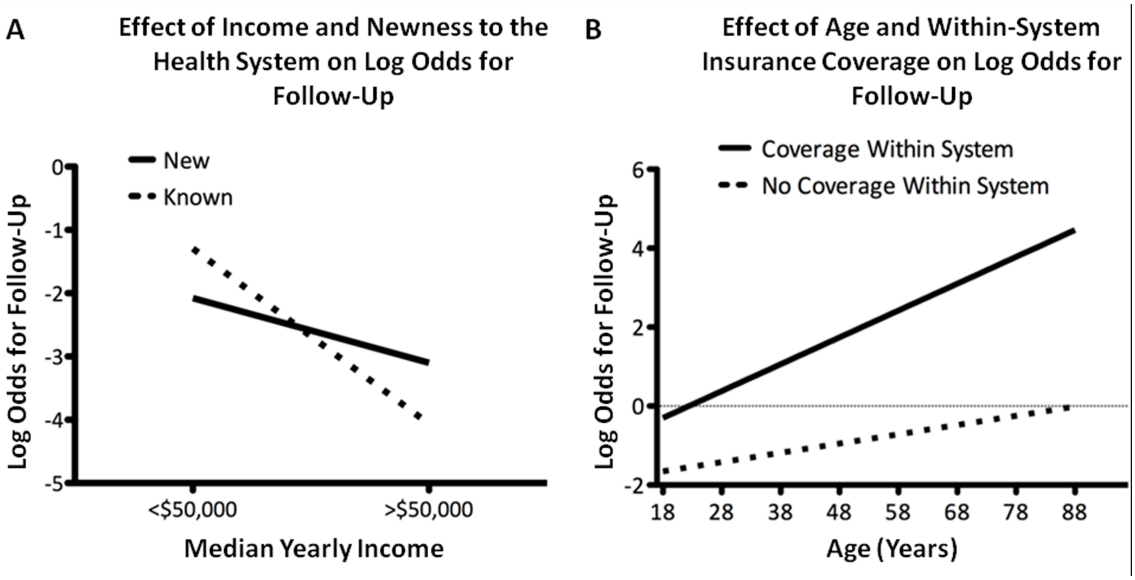


Figure 34: Significant cross interactions on logistic regression analysis.

6.5 Discussion

This study investigates factors affecting no-show in 464 consecutive patients presenting to the ED with a complaint requiring orthopaedic consult and clinic follow-up. Rigorous multivariate statistical analysis reveals several important orthopaedic-related factors and demographic variables which are predictive of no-show to clinic.

Orthopaedic Factors

First, the type of splinting utilized was predictive of follow-up. Plaster splints, knee immobilizers, and alumifoam splints all decreased no-show. Plaster splints may

serve as a reminder to patients of their injury and/or increase the patient's perception of the gravity of their injury, making them feel that follow-up is more important. In addition, these splints limit range of motion, are burdensome, and are more difficult to remove. Perhaps in situations where a patient is at high risk for no-show, and a choice must be made between a splint versus a brace, the risk-benefit-ratio may fall towards placing a splint (e.g. a nondisplaced ankle fracture that could be placed in a fracture walker boot versus a splint prior to clinic follow-up). It was surprising that alumifoam finger splints and knee immobilizers increased follow-up. This may represent the type of injury that the patients sustained and may be an indicator of the level of pain or limitation caused by those injuries (e.g., ligamentous injury or finger fracture) compared to patients who did not require any bracing or received other types of bracing.
Second, different no-show rates were found based on the anatomic region of injury. The highest follow-up rates were seen with patella/leg/foot injuries and forearm/wrist injuries. These distal extremity injuries were generally fractures (e.g., distal radius and ankle fractures), many of which are limiting to mobility and weight bearing in that extremity. Spine consults showed the highest rate of no-show. This subgroup was not analyzed in detail, but the suspicion is that the no-shows in a discharged population likely represent lower acuity complaints such as very stable fractures, sprains and strains, or patients with chronic spinal complaints as compared to admitted patients (e.g. immediately operative spine injuries).

Third, we found that patients whose injury was incurred during a physical assault had a higher no-show rate. Other studies have shown that assault victims represent a high-risk population that is more likely to suffer additional traumatic injuries

(253). Patients who are discharged home in particular have higher post-injury disability than other injured patients (254). Given the high risk for no-show in this population, special measures could be employed to encourage follow-up such as pre-discharge counseling, text reminders, or placement of a more cumbersome splint if clinically appropriate.

Fourth, in our study it was clear that different orthopaedic providers had significantly different no-show rates. There were no differences between these providers and the other providers in terms of race, primary language spoken, gender, or level of residency training. It is unclear exactly what the two high-risk providers did differently, but it is certainly worth studying in further detail. As patient outcomes and patient satisfaction become a part of reimbursement strategies, this type of discrepancy will become more important to hospitals and individual providers.

Demographic and Historical Factors

In our orthopaedic cohort both morbid obesity and tobacco use were predictive of no-show. These may represent markers of people who tend to be non compliant and do not safeguard their health at baseline, so are less likely to follow-up.

Age has been found in several studies to be predictive of no-show, with younger patients having a higher no-show rate (243-247, 255). Our study found similar results, and this effect was more pronounced amongst patients with in-system coverage.

In addition to conferring primary and secondary prevention of chronic disease, we found that having a primary care home improved attendance at specialty follow-up. This may have been through direct consultation with the PCP about the orthopaedic injury, or indirectly by building the patient's trust in the healthcare system.

Patients new to the health system were less likely to obtain follow-up in this study, perhaps due to perceived barriers and complexity of the system, or perhaps due to prior established relationships with outside providers. It may be important to recognize how difficult it can be to orient patients to navigating a new system, particularly those patients who have been uninsured and have not regularly been seeking care elsewhere. New patients may benefit from additional counseling or resources explaining how to navigate the hospital system, in order to ensure new patients embrace the system as the source for all of their follow-up as intended.

The other significant factors in this study are interesting, but may be more specific to the public hospital population served where low-income, uninsured, and minority individuals with many socioeconomic barriers to care are overrepresented. In this study, non-citizens were more likely to follow-up than American citizens. Perhaps this is a function of the lack of alternative care options for non-citizens, or the relatively low socioeconomic status of the American citizens who seek care in the public hospital rather than in nearby private alternatives. While this population decreases the generalizability of the data, it also presents an important view of a cohort that will be increasingly represented in multiple care settings as they gain insurance through the ACA.

The study was retrospective and consisted only of chart review without any patient contact. This health system utilizes an integrated model of care with one insurance program providing access to the entire system and encourages patients to seek all of their care within this same system. However, without contacting the patients it cannot be determined whether they sought care within other health systems.

Furthermore, the patient's perspectives on barriers to attending follow-up care, such as lack of transportation, inconvenient appointment time, or dissatisfaction with ED care cannot be confirmed.

Some studies have found that giving patients an appointment prior to ED discharge improves follow-up rates (245, 256). In this study, there was no significant difference in no-show rates between patients who were given an appointment on the day of the ED visit versus not. However, while we initially planned to compare different methods of scheduling patient appointments to find the most effective method, this was not possible because the method and time of appointment scheduling was not clearly documented in this EMR. This has identified a new area for quality improvement within our system.

6.6 Future Directions

This study identified provider, system and patient factors which may be targeted for improvement at our own hospital, and several important factors which are predictive of no-show to clinic amongst orthopaedic patients in general. The results emphasize that the origins are multifactorial. As such, patient no-show should be viewed as a "dysfunctional system issue and not simply as a patient-centered deficiency" (248). This places an onus on providers and hospital systems to examine and improve any factors within their control. Going forward, we should develop applications to use the EMR and other technologies to help track and improve compliance across all populations in order to optimize management and outcomes (257, 258).

CHAPTER 7: DISCUSSION

The overarching theme represented in this body of work is the advancement of care of the orthopaedic patient, through both basic research and clinically-related scholarly projects. As previously stated, improvements in patient care can range from the investigation of basic mechanisms informing the field of cancer biology to human subjects research. Here, several studies were presented with diverse content and unique contributions to the advancement of patient care.

In chapter 2, evidence suggested that the periostin/TGFBI ratio has prognostic significance in a preclinical model and in human breast cancer specimen. *In vivo*, this ratio correlated with distant metastasis to the bones, a condition that falls under the scope of the orthopaedic surgeon. Interestingly, both the ratio of periostin/TGFBI in both the primary tumor and in mouse plasma correlated with bone metastases, and the metastases present were microscopic rather than macroscopic in nature. This suggests that the periostin/TGFBI ratio offers potential as biomarker for the early risk for bone micrometastases, possibly allowing earlier identification and systemic treatment of these patients. Given the convenience of blood testing, using the serum ratio of periostin/TGFBI as a prognostic indicator could be clinically relevant. However additional studies in a larger cohort of human patients will be needed to further validate these findings. One potential limitation to the use of the serum periostin/TGFBI ratio is

that serum levels of periostin can be elevated in other non-cancer conditions such as asthma (259), idiopathic pulmonary fibrosis (260), chronic kidney disease (261), and acute myocardial infarction (262) leading to decreased specificity of this potential laboratory test.

In chapter 2, evidence was also presented that losartan decreased primary tumor size and bone metastasis in an immunocompetent mouse model. While these results will need to be repeated and further investigated, this data does suggest promise in the use of ARBs in the treatment of breast cancer. Existing retrospective studies examining the use of ARBs have found mixed results in heterogeneous groups of patients, however one study suggested that ARB use was protective against breast cancer recurrence (49, 263). A future direction of this study could include a randomized controlled human trial utilizing losartan as an adjuvant treatment with standard chemotherapy. Losartan is already approved by the Food and Drug Administration (FDA) and has a known safety profile, which would make translating losartan into use as a cancer adjuvant a feasible endeavor.

Chapter 3 presented the paradoxical effects of periostin on tumor-associated macrophages. Importantly, periostin pre-treatment of macrophages led to inhibition of tumor growth *in vivo*. However, at the time of harvest, distant metastasis was not significantly different between treatment groups. Additional studies will be conducted in the laboratory to further investigate the mechanism via which periostin's effects on macrophages are mediated. At this time, little is known about periostin's role in regulating immune cell function, and there is much to investigate. As periostin's immunoregulatory functions are discovered, the best method for harnessing or

modulating this activity will also become more apparent. At this time, periostin appears to have an overall pro-tumor effect, but as more is learned about periostin, the role of periostin may become more nuanced. As an aside, this investigation highlights the importance of using immunocompetent models when investigating novel cancer treatments, as some medications or interventions can have paradoxical and unanticipated effects that may undermine the treatment intention.

Chapter 4 presented an evidence-based review of management and outcomes of patients with soft tissue sarcoma. NCCN guidelines were discussed, and limitations in currently available treatments were noted. Importantly, this chapter highlighted the identification of specific molecular targets in GISTs, leading to tailored therapy and improved outcomes. To achieve additional improvements in the outcomes of soft tissue sarcomas, continued basic, translational and clinical research will be needed to identify specific targets for RPS and extremity soft tissue sarcomas. Reviews such as this one are helpful in distilling the most salient points in the literature to help surgeons managing these patients.

Next, Chapter 5 discussed the case of a patient with a malignant pheochromocytoma bone metastasis necessitating prophylactic fixation. This presentation is unique for the orthoapedic surgeon to encounter, and this chapter provided a useful clinical algorithm. Reports such as this help to advance the care of orthopaedic patients by raising awareness of these problems and sharing useful management principles.

Lastly, chapter 6 presented the findings of a retrospective study investigating the factors affecting orthopaedic patient follow-up in clinic. Interestingly, when patients

were seen in the emergency department, the orthopaedic resident who was consulted had a significant association with whether or not that patient would subsequently return for follow-up. This finding highlights the importance of the patient-physician relationship. Even with the most advanced science and treatment modalities, excellent care of patients ultimately depends on a relationship between a patient and a provider. Furthermore, socioeconomic factors and injury variables also were significantly associated with follow-up, and this study may help providers identify patients who are at risk for loss to follow-up.

Overall, this body of work contributes to the advancement of the care of orthopaedic patient on multiple fronts, identifying challenges and opportunities from the system-level of the provision of care down, to using evidence-based guidelines in management patients, to the investigation of potential treatments for cancer.

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