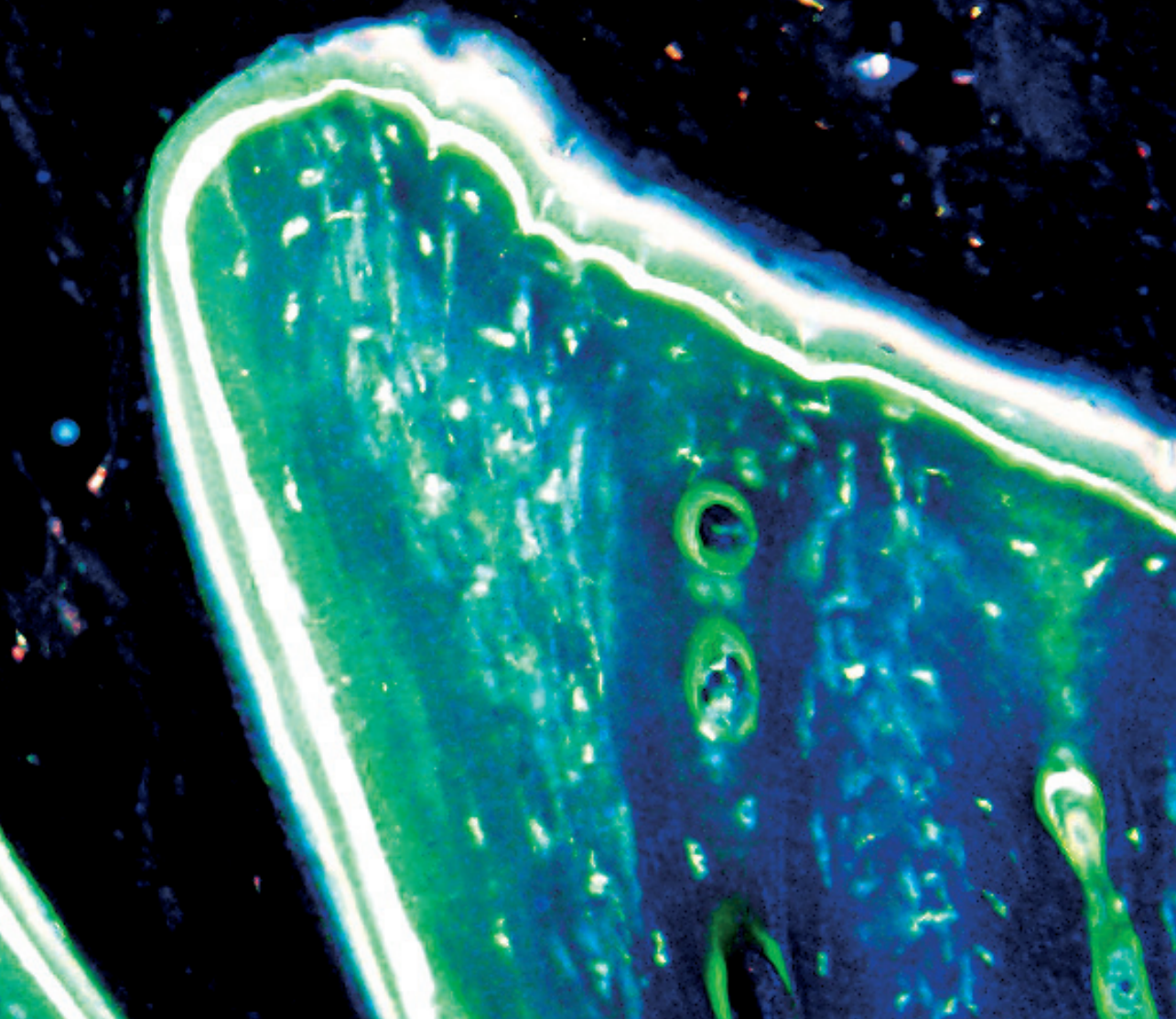


LIVING BONE AND JOINT ALLOTRANSPLANTATION

An experimental journey

Rudolph Henricus Houben



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VRIJE UNIVERSITEIT

Living bone and joint allotransplantation

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ter verkrijging van de graad Doctor
aan de Vrije Universiteit Amsterdam
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prof.dr. A.Y. Shin

Righty Tightly Lefty Lucy

Best memorable motto of the Microvascular Research Laboratory Mayo Clinic
in memorial of Lucy and the other animals

Dedicated to prof.dr. A.T. Bishop
To my grandmother and father; Truus and Dolph Westerbeek

TABLE OF CONTENTS

PART 1

Chapter 1	Introduction and Outline of the Thesis	11
Chapter 2	Vascularized bone grafts, a closer look at the free fibula flap for lower extremity reconstruction	31
Chapter 3	Combined massive allograft and intramedullary vascularized fibula as the primary reconstruction method for segmental bone loss in the lower extremity: a systematic review and meta-analysis	55

PART 2

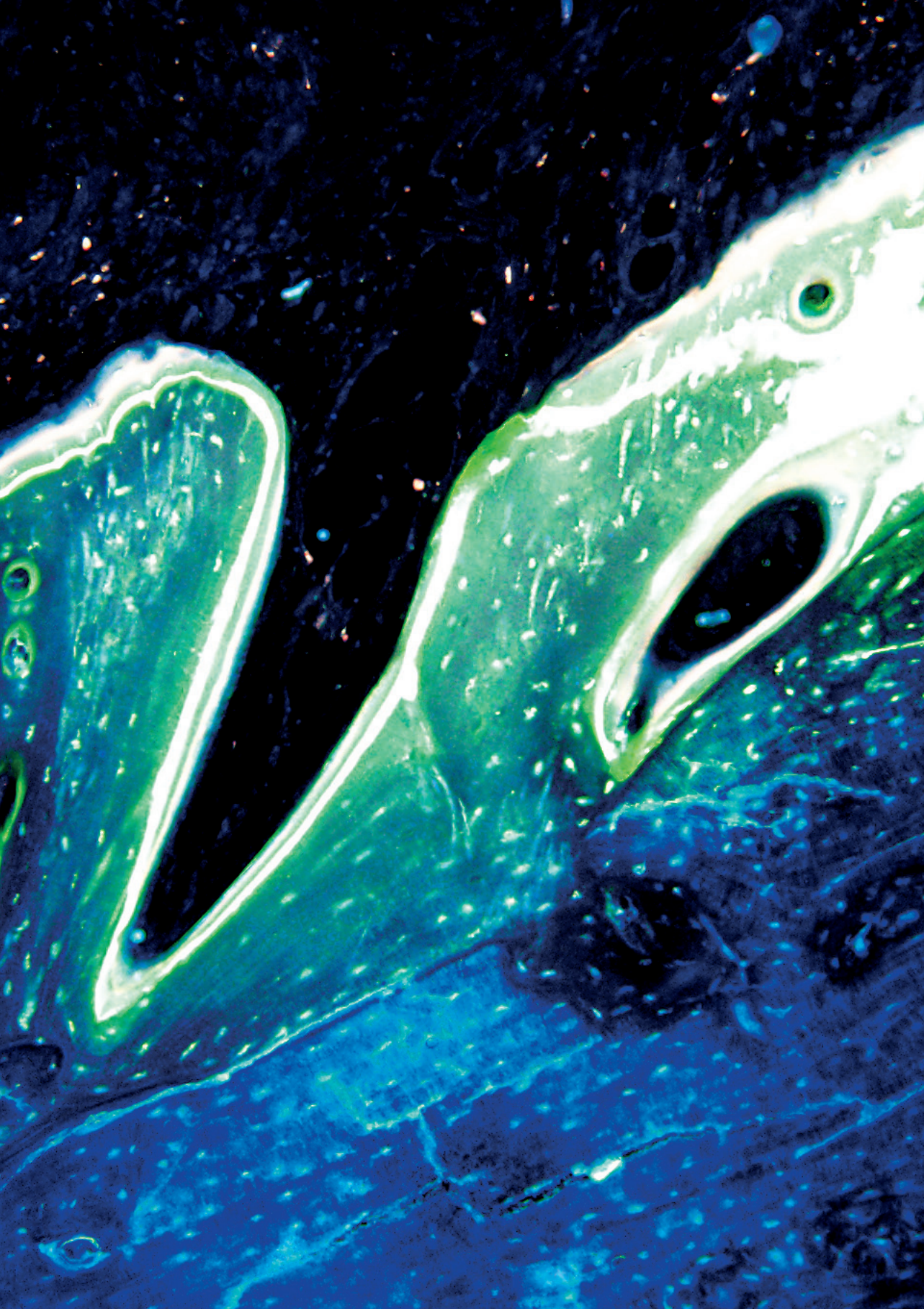
Chapter 4	Outcomes of vascularized bone allotransplantation with surgical induced autogenous angiogenesis in a large animal model: bone healing, remodeling and material properties	75
Chapter 5	Neo-angiogenesis, transplant viability and molecular analyses of vascularized bone allotransplantation surgery in a large animal model	95
Chapter 6	Transplant chimerism in porcine structural vascularized allotransplants	115
Chapter 7	Autogenous arteriovenous bundle implantation maintains viability without increased immune response in large porcine bone allotransplants	134

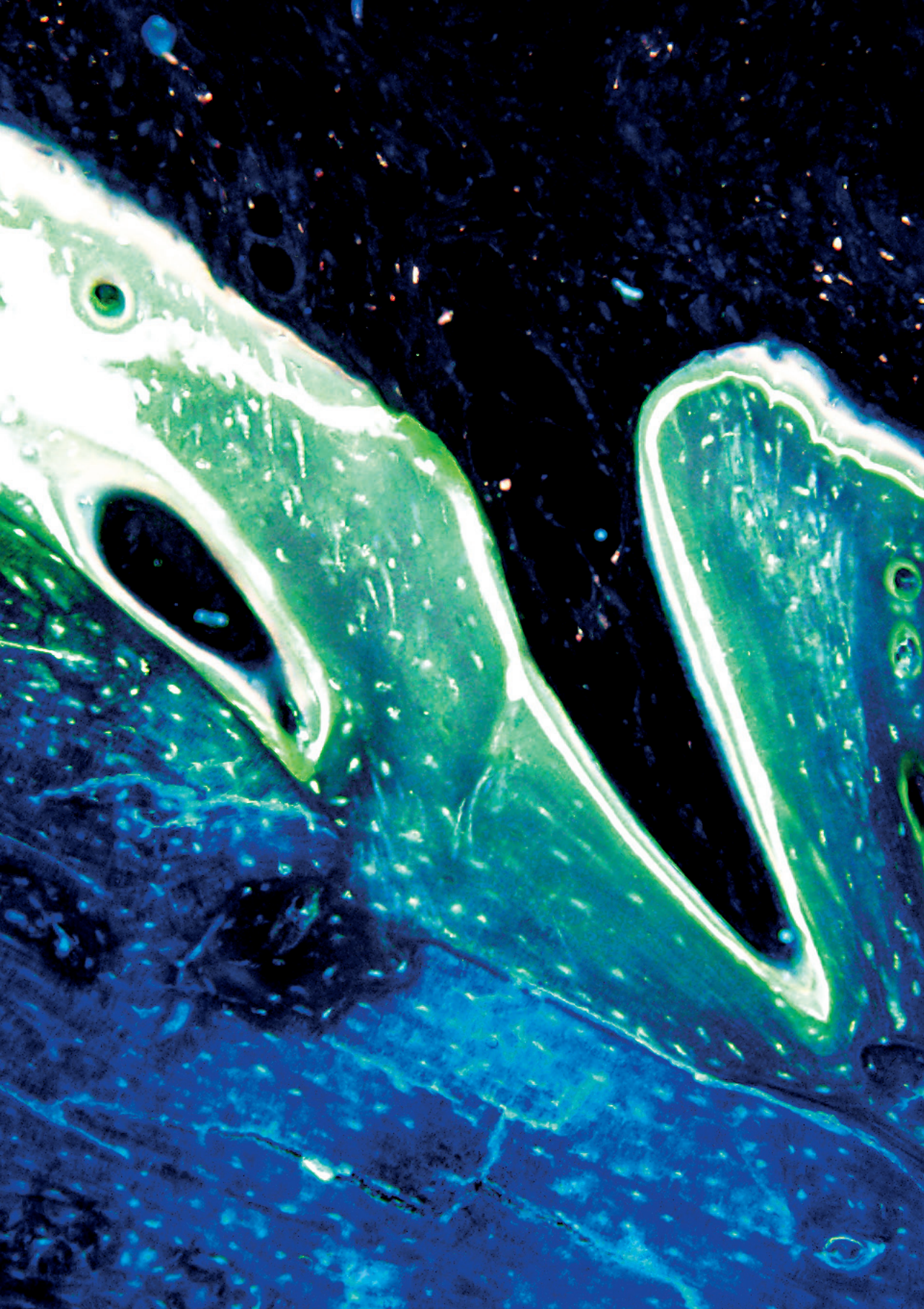
PART 3

Chapter 8	A new porcine vascularized whole knee allotransplantation model: anatomy, surgical technique and improvements	155
Chapter 9	Summary and general discussion	173
Chapter 10	Summary in Dutch (Nederlandse samenvatting)	185
List of abbreviations		192
Curriculum Vitae		194
PhD Portfolio		196
Dankwoord / Credits		200

PART

1





1

CHAPTER

Introduction:
background and significance,
aims and outline of thesis

The Clinical Problem: segmental bone and joint loss

Segmental loss of bone and/or joint in the appendicular skeleton results from limb-sparing resection of primary bone tumors (3450 cases/year in 2018)^[1], metastatic tumors, severe complex trauma, infection, congenital deficiency or failed primary reconstruction. Frequent complications occur with current available reconstructive methods, including cryopreserved bone allografts, vascularized bone autografts, prosthetic replacement, reimplantation of autoclaved tumor bone, bone transport, or a combination of vascularized autografts with massive allograft as discussed below. Future reconstructive methods may use a different approach by reconstructing the defect with “like for like” living tissue while minimizing complications.

Cryopreserved bone allograft (CBA) reconstruction

Reconstruction of segmental defects with a CBA provides immediate stability and bulk when combined with rigid internal fixation. They are widely used since they are easy to size and shape to match the defect. CBAs do not contain viable cells due to the process of freezing or irradiation, which diminishes the immunologic response. Therefore, bone healing occurs by creeping substitution. This is a slow and incomplete process, which is mostly seen at the host/allograft junction and periosteal surfaces. Fifty percent of the lamellar bone remains necrotic over time, with resorption of the CBA as result. Therefore, significant weakening results at 6-12 months after reconstruction^[2]. Although CBAs do not contain viable cells, a host immune response occurs which includes the development of anti-HLA antibodies^[3, 4]. Clinical outcomes of CBA reconstruction are often problematic with high incidences of non-union (14%), stress fractures (19%) and infection (12.8%)^[5, 6]. Fracture and non-union require revision since they lack the ability to repair and remodel^[7]. Infection is a catastrophic complication. Due to the avascular status neither the immune system nor antibiotics will reach the infected bone. ischemia-reperfusion requires allograft removal and has a significant risk of recurrence when revised^[4, 5].

Vascularized bone autograft (VBG) reconstruction

Transfer of living bone has been a reconstructive method since 1905^[8-10]. The first free vascularized fibula transfer was described in 1975^[11]. Other donor sites included rib, iliac crest, scapula, radius, femoral condyle and trochanteric bone^[12]. VBGs are indicated in large segmental bone defects or can also be used in smaller bone defects where other non-vascularized options are likely to result in “biologic failure”. Examples include persistent non-union after CBA reconstruction, scarred or irradiated soft tissue beds, and the need to re-vascularize avascular bone in cases of radionecrosis^[13-16]. Other indications include tissue loss requiring composite bone/soft tissue reconstruction, joint arthrodesis, congenital pseudarthrosis and need for longitudinal bone growth requiring physeal transfer possible with the inclusion of the proximal fibular epiphysis and physis^[16-18]. VBG remain viable due to maintenance of its endosteal and/or periosteal circulation provided by microvascular repair of its vascular supply. Therefore, osteocytes and other cellular components remain viable. No creeping substitution occurs, obviating osteocytopenia and resulting in improved strength and healing capacity compared to other methods^[15, 19]. Up to 80% of the structural VBG will show significant hypertrophy over time in response to stress loading by 24 months following reconstructive surgery^[20, 21]. In many instances, VBGs require protection

from mechanical loads during the hypertrophic process because they are often poor structural replacements for metaphyseal defects and grossly mismatch the shape and size for humerus, femur and tibia diaphysis [22-24]. Limited donor site availability, donor site morbidity, and size/shape mismatch at the recipient site are therefore frequent problems with VBGs [16, 22]. Due to the enhanced healing potential, a primary union rate of 61% has been reported [16]. Further evaluation of the free fibular flap as a reconstructive method will be discussed in chapter two. Currently, the use of vascularized bone autografts remain the gold standard in the reconstruction of large segmental defects since the use of vascularized composite allotransplantation requires the use of life-long immunosuppression for non-life-critical tissue allotransplantation.

Other reconstructive methods

Diaphyseal or periarticular defects can also be reconstructed with custom endoprosthetic replacements that allow immediate restoration of function [25-28], but have limited longevity. Complication rates for prosthetic reconstruction range from 33% to 100% and are most serious requiring revision or amputation due to mechanical failure or infection [29, 30]. Allograft/prosthetic hybrids have generally failed due to implant loosening, fracture or separation [25, 31, 32]. The re-implantation of resected tumor bone has been described as a reconstructive method after devitalizing the tumor bone by autoclaving [33, 34], pasteurization [35, 36], irradiation [37] or cryotherapy [38]. Re-use of devitalized tumor bone carries the risk of incomplete tumor death and an incidence of a stress fracture and infection similar to or even greater than CBAs [30, 34]. Bone transport is best suited for modest segmental bone defects, with healing times of 1-3 years, [39] and is associated with pin-tract infections, soft tissue problems, and non-union at the docking site [40, 41]. The induced membrane technique, also known as the Masquelet technique [42], is a two-stage procedure utilizing a cement spacer in the first stage and a mixture of autologous cancellous bone in the second stage. The downsides of this two-stage reconstruction are the requirement of a substantial time delay allowance of full weight-bearing, along with associated complications as infection, resorption, and non-union [43]. A combination of a vascularized fibula flap with CBA does provide the benefit of enhanced healing and hypertrophy capacity of VBG with the strength and bulk of a CBA in a one-stage reconstruction. This technique has been known as the Capanna technique [44] and is widely used in the reconstruction of segmental bone defects after primary tumor resection. The Capanna technique is especially suitable for the younger patient after primary tumor resection in the lower extremity [23]. It results in good long-term outcomes with relative high complication rates, reintervention rates and requires a donor site [23, 43-47]. This technique will be further discussed in Chapter 3.

Living bone and joint allotransplantation

A future alternative for skeletal reconstruction may be bone and/or joint vascularized composite allotransplantation (VCA). VCA is defined as the transplantation of living allogeneic tissue from an organ donor and microvascular repair of its allogeneic vascular supply to maintain transplant viability. Living bone and joint allotransplantation combine the advantages of CBAs with the enhanced healing and remodeling potential of autologous VBGs, minimizing the risk of late stress fractures, resorption and infection [12, 48]. Transfer of living allogeneic tissue will closely match the defect size, shape and tissue type, and would eliminate donor site morbidity. Current multi-organ donor programs have the ability to identify and match the right donor and recipient. Bone is relatively ischemia-tolerant, and tissue harvest may therefore take place after critical organ harvest has been completed and bone tissue viability can be maintained for up to 5 days in cold storage [49-53].

First clinical experience

In VCA and organ transplantation, rejection, morbidity, and mortality resulting from efforts to modulate the immune system are major issues. Cell-mediated and humoral responses develop in 3-5 days after VCA transplantation [54] and cause subsequent rejection if immunosuppression is not administered [55-58]. While microvascular surgery makes VCA technically feasible, to date VCA requires the use of life-long immunosuppression (IS). Internationally more than 100 upper extremity transplantations and 30 face transplants have been performed with an incidence of acute rejection exceeding 80% [59]. Before 2010 (first facial transplant), 49 hand transplantations have been performed internationally. Complications included acute rejection (85%), opportunistic infections (88%), and metabolic or drug-related problems in 70% [60]. Twenty-four percent of the hand transplantations failed, with amputation or patient death as a result. Clinical experience with bone and joint VCA is limited. In 1990 the first vascularized allogeneic femoral diaphysis was transplanted in a human without the use of IS, since donor and recipient were ABO blood system and Human Leukocyte Antigen (HLA) matched [61]. Later in 1996, a congenital pseudarthrosis was treated with a vascularized allogeneic fibula flap from mother to daughter using IS, constituting the first successfully-healed VCA described in the literature [62]. A German group has reported the largest cohort of clinical bone and joint VCA. They reported transplantation totals of three femoral diaphysis and five whole knees in 2000 [63]. Most of the clinical VCA reconstructions to date have eventually failed due to infection, acute rejection, or chronic allotransplant vasculopathy [63-71].

Immunology of vascularized composite allotransplantation

Vascularized composite allotransplantation has revolutionized the reconstructive options for the most challenging tissue defects. For example, the clinical successes achieved in hand and face allotransplantation are based on the knowledge gained in solid organ transplantation. The immunologic reaction of the body against allogeneic tissue is the result from the interplay between the innate (non-specific) and adaptive (specific) immune response. The innate immune response is largely mediated by macrophages, dendritic cells, neutrophils, natural killer cells, and the complement system. These cells respond to 'danger' which can be induced by surgical trauma, infection, ischemia-reperfusion injury, mutated cells, and foreign tissues. Activated innate

cells produce chemokines which recruit leukocytes and antigen-presenting cells (APCs) to the transplant site. The adaptive immune response is mediated by antigens presented by APCs (dendritic cells, macrophages), which undergo either direct or indirect T-cell allorecognition. At the same time, APCs from the allotransplant migrate from the allotransplant into the recipient and can therefore be involved in the adaptive immune response. The direct pathway is responsible for most acute rejection episodes, where the indirect pathway is responsible for chronic rejection. The direct pathway allorecognition is activated by MHC class II and I alloantigen's recognized by CD4 and CD8 T cells directly presented by donor antigen-presenting cells (APCs, mostly dendritic cells). In the indirect pathway, the MHC antigens are first internalized by the APCs and thereafter presented as peptide fragments, and recognized by CD4 and CD8 T cells [72]. After activation of the T cells through binding of the antigen, they undergo a process of signal transduction, amplification, and production of transcription factors. These lead to proliferation and secretion of immunoglobulins and cytokines [73]. Reports of B cell responses or antibody-mediated rejection of VCAs are limited [60].

Drug therapy in VCA

As discussed above, in clinical practice, suppression of VCA rejection is achieved through a comprehensive multi-level immunosuppressive drug therapy. Current immunosuppressive regimens are similar to those of solid organ transplantation, including a combination of calcineurin inhibitors (tacrolimus), antiproliferative agents (mycophenolate mofetil), and different dosing regimens of corticosteroids [74]. This blocks the formation, stimulation, proliferation, and differentiation of lymphocytes [75-77]. Prevention of musculoskeletal tissue transplantation rejection is even more problematic and requires 2-3 times greater immunosuppressive doses [78]. Inadequate drug treatment results in rejection and causes damage to the vascular endothelium, with subsequent increased vascular permeability and activation of leukocytes [79]. Thereafter, thrombosis of the allogeneic circulation occurs resulting in tissue death. Long-term drug therapy carries significant risks, including opportunistic infections, delayed wound healing, malignancy, metabolic diseases and end-organ toxicity [60, 74, 80, 81]. Thus, life-long IS presents an ethical dilemma in non-life-critical tissue transplantations.

Tolerance induction and allotransplantation

Induction of donor-specific tolerance is considered the 'holy grail' of organ transplantation, providing a method of maintaining long-term transplant viability without the use of drug therapy. The concept of engraftment of donor bone marrow cell in host tissue (mixed chimerism) to induce donor-specific allograft tolerance was first described in the 1950s [82, 83]. Tolerance induction by the introduction of mixed chimerism is believed to be of importance by many researchers [84-86]. This theory has therefore been tested in many experimental settings with various outcomes. Previous research from our laboratory used skin-grafting as a means for evaluation of immune competence and for demonstrating the absence of donor-specific tolerance after bone only VCA [87]. Additionally, donor-specific tolerance has rarely been achieved clinically and is therefore controversial [88]. Another proposed approach to induce tolerance is the use of regulatory T cells, thus suppressing the rejection response. This has led to drug-free transplant survival in three patients for a limited period of time [89, 90]. Thymic clonal deletion of responding immunocytes, use of costimulatory blockade [91, 92], low-dose radiation, infusion of antilymphocyte serum combined

with IS ^[93-97], and infusion of donor bone marrow-derived cells combined with antilymphocyte serum ^[98] are other proposed methods of inducing tolerance in allotransplantation for some period of time. All methods have serious drawbacks as the tolerant state is not maintained and graft-versus-host disease (GvHD) can occur which is potentially lethal ^[99-101]. Complete ablation of the recipient's hematopoietic system by whole-body irradiation prior to allogeneic engraftment may minimize GvHD but is also potentially lethal ^[102]. The complications associated with tolerance induction are as onerous as those of immunosuppression. Therefore, current techniques to maintain allotransplant viability by tolerance induction or drug-therapy are impractical for clinical use. Other methods to maintain transplant viability without drug-therapy or tolerance induction need to be considered to make VCA a success as a possible alternative reconstructive method.

Innovation

Bone and/or joint VCA acceptance may also be facilitated by transplant chimerism, the replacement of the allogeneic bone with recipient-derived osteocytes ^[48, 103-105]. This is a novel method of maintaining bone and joint VCA viability without the need for either life-long immunosuppression (IS) or tolerance induction. The method replaces the allogeneic endosteal circulation with a recipient-derived neoangiogenic circulation. This is accomplished by the implantation of an autologous (recipient-derived) arteriovenous (AV) bundle into the intramedullary space, together with microvascular repair of the allogeneic nutrient vessels. Only short-term immunosuppressive drug therapy is required. During the short-term IS period, the implantation of autogenous AV bundle results in the rapid development of an autogenous neoangiogenic circulation. After cessation of the IS, the allogeneic pedicle will eventually thrombose due to rejection. Angiogenesis or vasculogenesis is the biologic formation of new capillary vessel, mediated by growth factors, cell interactions, and proteolytic enzymes that modify molecules on cell surfaces and extracellular matrices ^[106]. Surgical angiogenesis is the transfer of vessels or well-vascularized autogenous tissue into an area of relative hypovascularity ^[107-110]. Surgical angiogenesis for the induction of a neoangiogenic circulation in autogenous bone was first described in a canine tibia model ^[109]. The implantation of AV bundles for the induction of new bone formation and revascularization have been shown in autograft ^[15, 108, 111, 112], allograft ^[110, 113-117], and xenograft bone ^[118, 119]. Clinical application of surgical angiogenesis has been described in avascular necrosis of the talus ^[109], Kienbock's disease ^[120, 121], scaphoid non-union due to avascularity ^[122-124], and prefabricated bone flaps ^[125]. Multiple small animal studies have been conducted on the use of surgical angiogenesis in maintaining bone and joint VCA viability. All demonstrate living bone allotransplants survive by surgical angiogenesis alone after cessation of the immunosuppression. The neo-angiogenic circulation, promotes new bone formation, maintains bone material properties and limb function^[87, 110, 118, 126-134]. Multiple lineage studies evaluated areas of new bone formation and active remodeling. These studies demonstrated repopulation of the allotransplant by recipient-derived osteocytes over time which indicates transplant chimerism ^[134-136].

Yucatan miniature swine as large animal transplantation model

The majority of the VCA research conducted by the microvascular research laboratory at Mayo Clinic has been performed in small animal models. Laboratory rats and rabbits have been most commonly used, due to their defined immunology, easy handling, and availability. These studies have shown promising results in maintaining VCA viability by surgical angiogenesis and short-term immunosuppression [87, 105, 126-136]. For translatable results, a porcine model is of pre-clinical importance since they have distinct advantages for allogenic tissue transplantations research. Their size, anatomy, physiology, and immunology are well known and comparable to man. Most importantly, both blood type and the major histocompatibility haplotypes (swine leukocyte antigen, SLA) have been well defined. SLA haplotypes can be determined by pre-operative DNA sequencing [137-139]. This allows transplantation over a major mismatch in histocompatibility while animals are matched for blood type, size, and age. Evaluating transplantation chimerism is possible if a sex-mismatched experimental set-up is used [140]. Regular blood draws allow long-term monitoring of systemic immune responses after transplantation by cytokine detection and blood cell counts. For bone and joint, orthotopic reconstruction of a segmental defect uses surgical techniques and implants identical to clinical use. Their physiology, including the rate of new bone formation, is nearly identical to man [141]. A number of VCA studies have taken place using miniature swine, including skin, muscle, bone, bone marrow and composite knee joint allotransplants [56, 142-148]. All of these studies used an subcutaneous inguinal pocket in which the allotransplant was transplanted. Orthotopic allotransplantation of bone and joint is unknown in large animal models. A large animal tibia defect model has been developed in Yucatan miniature swine for more translatable results [149, 150]. This Yucatan miniature swine model has proven to be a great asset. This pre-clinical model allows orthotopic microsurgical transplantation of bone VCA, long-term monitoring of the transplanted tissue and host immune response while resulting in minimal morbidity for the animal [151].

Significance

Segmental bone or joint loss is a serious and challenging clinical problem after primary or metastatic bone tumor resection, severe trauma, infection, congenital pseudarthrosis, or failed primary reconstruction. Current methods used to reconstruct these defects have significant problems. Cryopreserved bone allografts (CBAs) are widely used since they are available in the right size and shape, and they provide immediate stability and sufficient bulk to the reconstruction. Complications associated with this type of reconstruction include non-union, infection, and late stress-fractures due to their avascular status. Vascularized bone autografts (VBGs) contain their own intrinsic blood supply due to microvascular repair of the nutrient vessels. Osteocyte viability is therefore maintained, resulting in improved strength, faster union times, and hypertrophy potential. Despite these favorable properties, due to the lack of donor sites VBGs often mismatch the defect in size and shape. Additionally, VBGs are associated with donor site morbidity. Due to the complications and limitations of current reconstructive methods, innovation is needed. Microsurgical transplantation of living allogenic bone and/or joint may be a future alternative for the treatment of segmental defects. Currently, VCAs require multi-level immune modulation to prevent rejection of the allotransplant. Immunosuppressive regimens for VCA are similar to those in solid organ transplantation. Complications of life-long IS include, opportunistic infections, malignancy,

organ toxicity, wound healing problems, metabolic diseases, and high costs. Therefore, life-long immunosuppressive therapy is contraindicated in oncological patients and presents an ethical dilemma wherein it is questionable to use life-long IS for non-life critical transplantations. In the past years, a novel method has been developed maintaining allotransplant viability without the need for life-long immunosuppression or induction of tolerance. Surgical angiogenesis combined with short-term IS may maintain VCA viability and has shown potential in small animal models. This novel method is therefore the key focus of our research. We will investigate methods of maintaining bone and joint VCA viability with short-term IS and surgical angiogenesis in a pre-clinical porcine model. Through this investigation, we will refine our understanding of bone healing, remodeling, mechanical bone properties, pedicle patency, AV bundle patency, local and systemical immune response, and osteocyte lining (transplant chimerism). Further, we will demonstrate the feasibility of the method to transplant allogeneic vascularized composite whole joints in a porcine model. An evaluation of clinical literature of VGBs and combined VBG with CBA reconstruction is also provided (Chapter 2 and 3).

Aims and outline of the thesis

The overall goal of this thesis is to microsurgically transplant living allogeneic bone and joints and maintain viability without the need for life-long immunosuppression (IS) or tolerance induction in a pre-clinical model. In this thesis we further evaluate a novel method of maintaining VCA viability by switching the circulation of the allotransplant from allogeneic to autogeneic in a large animal model. This may be accomplished by combined microsurgical allotransplantation and simultaneous development of a new autogeneic blood supply in the allotransplant. Surgical implanted autogenous arteriovenous (AV) bundles will promote autologous neo-angiogenesis during the short-term immunosuppressive period. As this recipient-derived circulation is non-immunogenic, allotransplant viability will be maintained while the allogeneic circulation will thrombose due to rejection.

Hypothesis 1: In clinical practice, segmental bone defects are best reconstructed with a vascularized autograft combined with or without a massive cortical allograft.

Aim 1: To study the history, indications, contra-indication, surgical technique and clinical outcomes of the vascularized fibula flap at the Mayo Clinic (Chapter 2).

We will perform a literature search within Mayo Clinic published articles on the vascularized fibula flap for the reconstruction of segmental defects.

Aim 2: To evaluate the clinical outcomes of a combined allograft and vascularized fibula flap reconstruction (Chapter 3).

We will perform a systematic review on the use of combined massive allograft with intramedullary vascularized fibula flap for the reconstruction of large bone defects in the lower extremity.

Hypothesis 2: Bone VCA viability can be maintained in a large animal model using surgical angiogenesis and short-term immunosuppression without tolerance induction or other permanent immune modulation.

Aim 3: To successfully reconstruct segmental tibial bone loss by microsurgical transplantation of tibial VCA's + autologous surgical angiogenesis in Yucatan mini pigs (Chapter 4).

We will orthotopically transplant bone-only VCAs across a major histocompatibility barrier using sex-mismatched Yucatan miniature swine, implanting an AV-bundle within the medullary canal to induce recipient-derived angiogenesis (surgical angiogenesis). Two weeks of immune suppressive triple therapy will be used. We will implant a patent AV-bundle in group 1 and a ligated AV bundle in group 2 as a no angiogenesis control. In both groups a microsurgical repair of the nutrient vessel is achieved in the recipient combined with ridged internal fixation of the allotransplant.

Aim 4: To study allotransplant viability after cessation of the immunosuppression (Chapter 5).

We will histologically evaluate allotransplant sections and quantify osteocyte viability 20 weeks after transplantation. With the use of Micro-CT analyses, we will quantify a micro angiography of the allotransplant as a measure for neo-angiogenesis.

Aim 5: To study allogeneic pedicle patency as a function of time (Chapter 4).

We will use Doppler ultrasound during the survival period of the animals to check the allogeneic pedicle patency. We will measure the systolic and diastolic peak velocities in m/s.

Aim 6: To measure bone healing, ambulation and bone material properties after transplantation and cessation of immune modulation (Chapter 4).

We will observe weight bearing on the operated leg after transplantation on daily basis, measure bone healing scores by taking radiographs at evaluation time points (0,2,4,6,10,20 weeks), and will use biomechanical evaluation methods as axial compression testing and cyclic reference point indentation to measure bone material properties. Additionally, with the use of Micro-CT analyses, we will measure the bone micro architecture, bone mineral density, and quantify union 20 weeks after transplantation.

Aim 7: To study histological changes in the allogeneic pedicle, 20 weeks after transplantation (Chapter 7).

We will harvest, embed, cut and stain (Elastica-Van Giesson) the allogeneic pedicle after sacrifice of the animal. Thereafter we will histologically evaluate the microscopic changes of the allogeneic pedicle.

Aim 8: To study the underlying systemic and local immune responses to bone VCA with and without autologous AV bundle implantation (Chapter 7).

We will obtain peripheral blood at evaluation time points (0,1,2,4,6,10,20 weeks) to monitor the systemical immune response by blood cell counts and multiplex cytokine analyses in both intervention groups.

CHAPTER 1

Aim 9: To measure the extent of new bone formation and correlate bone formation to measures of angiogenesis (Chapter 4).

Areas of new bone formation will be identified by fluorochrome labeling. We will quantify the amount of new bone formation and resorption by histomorphometric analyses.

Aim 10: To study gene expression associated with the formation of new bone and a neo-angiogenic circulation (chapter 5).

We will quantify gene expression in the allotransplant by RNA extraction and subsequent reverse transcript qPCR analyses for multiple genes associated with bone formation, resorption, remodeling and neo-angiogenesis.

Hypothesis 3: New bone formation in transplanted allogeneic bone is the result of transplant chimerism

Aim 11: To measure the lineage of osteocytes in areas of new bone formation after transplantation and viability (chapter 6).

Transplanted (male donor bone) specimens removed from female recipient animals will be studied. Areas of new bone formation will be identified by fluorochrome labeling. We will micro dissect these areas of new bone formation by use of Laser Capture Microdissection (LCM) technologies and extract g-DNA. With the use of real-time qPCR we can amplify the genomic DNA and quantify the SRY gene (Y-chromosome specific) in areas of new bone formation. If no SRY can be amplified, the new bone formation is recipient-derived.

Aim 12: To measure repopulation of the allotransplant by recipient derived cells due to autologous AV bundle implantation (Chapter 6).

Complete sections of the allotransplant will be pulverized, and g-DNA and RNA extracted to quantify the SRY gene and RPL-4 to test the repopulation rate and viability of the remaining male donor cells.

Aim 13: To record levels of chimerism in peripheral tissues and determine if any systemic chimerism may have resulted in a state of donor specific tolerance (Chapter 6).

Liver and spleen specimens will be obtained 20 weeks after bone-only VCA transplantation and extraction of g-DNA. Real-time qPCR will be used to quantify the relative copy number of SRY gene (Y-chromosome specific) compared to RPL-4. This determines if there was a level of systemic chimerism after bone only VCA.

Hypothesis 4: Surgical angiogenesis will preserve viability, permit healing and maintain articular function of whole joint allotransplantation

Aim 14: To successfully reconstruct complete loss of a whole knee joint by microsurgical transplantation of a whole knee joint VCA in a porcine model (Chapter 8).

We will orthotopically transplant a whole porcine knee VCA with microsurgical anastomosis. Simultaneously, an autogenous cranial tibial AV bundle is implanted in the tibia and a muscular branch of the femoral biceps is implanted in the femur. The complete reconstruction will be covered by a pedicled gracilis muscle flap. Short-term immunosuppression will prevent rejection of the allogeneic pedicle during neo-angiogenesis occurring from the AV bundle. The entire knee joint VCA will be internally fixed with an intramedullary fixator system.

Aim 15: To study allotransplant viability, healing and biomechanical properties of bone and joint tissues (Chapter 8).

We will evaluate the viability of femoral and tibial bone and hyaline by histology and correlate these findings with mechanical properties, joint movement, weight bearing, and neo-angiogenesis measured by micro-CT.

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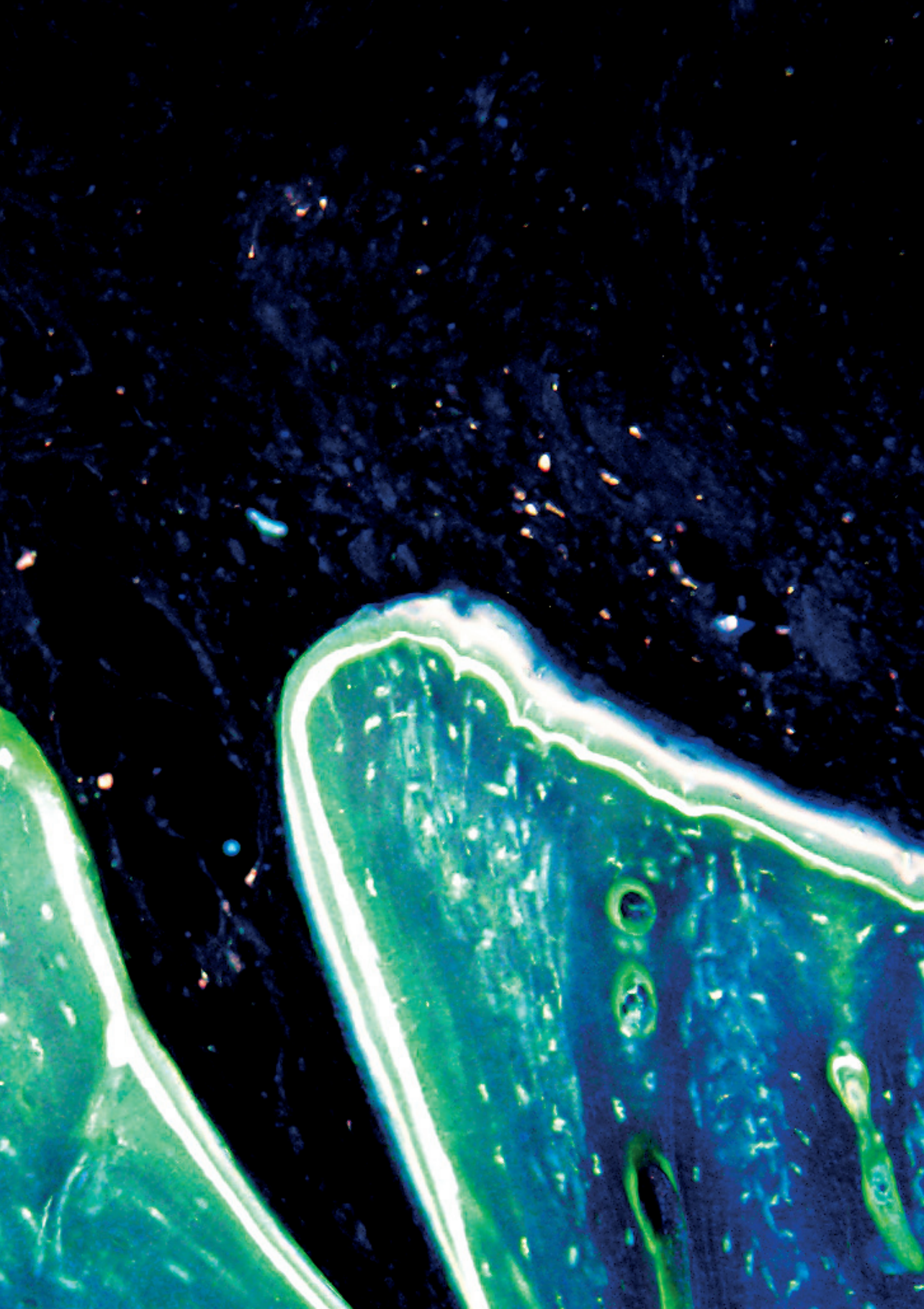
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2

CHAPTER

**Vascularized Bone Grafts,
a closer look at the free
fibula flap for lower extremity
reconstruction**

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The Clinical Problem

Large segmental bone defects cause significant disability in patients after limb-sparing surgery. Segmental bone loss is often the result of trauma, primary malignant tumor resection, meta-static tumor resection, infection, failed primary reconstruction, or congenital pseudarthrosis. Treatment of these large bone defects is a challenging problem. The eventual goal of limb-sparing surgery is to preserve limb function and prevent amputation. All current treatment options have their specific advantages and disadvantages.

The current state of treatment options

Although amputation remains an option for treatment of segmental bone loss, reconstruction of the bony defect is often to be preferred. Limb function can generally be restored when tissue loss for whatever reason spares critical neurovascular structures. Methods used to restore gaps in structural bone include vascularized bone transfer (commonly fibula or iliac crest ^[1-4]), bone transport, the Masquelet technique ^[5], prosthetic replacement, re-implantation of autoclaved tumor bone and structural and osteoarticular allograft bone, used alone or in combination with other methods. Allografts segments are frequently used to reconstruct large segmental defects. They are readily available and selected to match defect size and shape. With internal fixation, they provide desirable immediate stability. Over time they remain largely necrotic and are therefore susceptible to infection, non-union, and late stress fracture ^[6,7]. Bone transport will close only modest bone segmental gaps, with healing times of 1-3 years ^[8]. Custom endoprosthetic replacement provides immediate function, but with limited endurance ^[9]. The complexity of reconstruction by any technique does not surprisingly result in higher rates of complications than those of amputation. For example, prosthetic failure rate reports range from 33% to 100 %, mostly due to mechanical failure and/or infection, requiring revision or amputation ^[10-12]. Allograft/prostheses hybrids have also generally failed, with frequent implant loosening, allograft fracture, nonunion, or infection ^[13-15]. The re-use of autoclaved tumor bone risks incomplete tumor death and an incidence of a stress fracture and infection greater than cryopreserved allograft bone ^[11, 12].

History of the (Vascularized) Bone Grafts

Bone autografts, allografts, and vascularized bone grafts are currently widely used for the reconstruction of large segmental defects. The history of bone grafts is ludicrous and cruel. The use of bone grafts for the treatment of bone defects was first described in 1668 by a Dutch surgeon Job van Meekeren ^[16]. He described the first procedure of using a bone graft. This bone graft was derived from a dog's skull. He implanted the bone segment in a traumatic bone defect of a soldier's cranium. In the 17th century, this method was considered unchristian and the patient was excommunicated. For this reason, the soldier asked the surgeon Job van Meekeren to remove the bone graft, although by this time the bone graft was already healed. The idea of bone grafting has been invented by the Dutch scientist Antoni van Leeuwenhoek ^[17]. The French scientist Ollier experimented with bone grafts on rabbits and dogs and concluded that bone autografts are viable. He recognized that separated bone fragments could survive in a suitable environment without periosteum ^[18]. In more recent decades, several experimental and clinical

studies have led to the clinical usefulness of bone autograft and allograft reconstructions. However, careful analyses of the results suggest that a variety of problems are associated with this type of reconstruction as mentioned in the previous paragraph. Transplantation of a bone segment immediately revascularized by vascular repair was thought to solve these problems. The insertion of a piece of living bone that will exactly fill the gap and will continue to live without absorption has been attempted in 1891 by Dr. Phellps. He conducted a bizarre experiment in which he transplanted a piece of living bone from a dog as an interposition graft in a defect of the tibia of a boy. After transplantation, the patient and the dog were attached for 15 days. The transplantation failed and was removed after five weeks ^[19]. The desirability of a living bone graft above a non-vascularized allograft has been emphasized by several early investigators who used pedicled autologous fibula grafts for tibial reconstruction ^[20-22]. Reconstruction of a long defect may be accomplished by a mode of healing similar to that of segmental fracture rather than the usual more lengthy process of graft incorporation ^[23]. Pedicled bone grafts as rib, clavicle, iliac crest, scapula, humerus, radius, greater and lesser trochanter, medial femoral condyle, pisiform, and second metacarpal have been developed ^[24-33].

Free vascularized bone autografts involve the isolation of a bone segment on the nutrient vascular pedicle and its transfer to a distant site by microvascular anastomosis ^[23, 34]. This allows for the survival of osteogenic cells ^[35-37]. The microsurgical transfer of living autogenous tissue awaited the development of techniques and instruments for microvascular anastomosis. Due to improved microsurgical techniques and instruments, the development of free vascularized bone flaps was rapid ^[38]. Although the first procedure was performed by Ueba and Fujikawa in 1974 ^[39], microvascular free fibula transfer as a technique for salvage of lower limb skeletal defects was first reported in the literature in 1975 by Taylor et al. ^[40]. Since that time, the vascularized fibular bone graft remains the most common vascularized bone graft due to its, predictable vascular pedicle, mechanical strength, and potential for hypertrophy and growth ^[41]. The free microvascular fibula transfer has become an important tool in the armamentarium of the reconstructive surgeon dealing with the management of long bone defects and difficult non-unions.

Biology of Vascularized Bone Grafts (VBG)

Living bone has significant physiologic advantages to many other reconstructive methods in restoring limb form and function. Survival of osteocytes cannot be expected in autogenous bone depending upon diffusion of oxygen from surrounding tissue. This is particularly true in limb salvage surgery, where the transferred autograft is large and bulky. Vascularized bone flaps remain viable due to the maintenance of its endosteal and/or periosteal circulation provided by microvascular repair of its transplanted vascular supply [37, 42, 43]. Remaining viable and dynamic in its new site, a VBG does not undergo the gradual replacement of dead trabeculae by creeping substitution. Creeping substitution is a process, required for any non-vascularized or 'conventional' graft. In this process, osteoclasts precede the osteogenesis, with osteopenia and loss of mechanic strength as result [44-46]. This increases the risk of late stress fractures. Not only does the intrinsic blood flow of a VBG maintain strength, but it also increases and improves the rate of healing. It is capable of responding to applied stress by remodeling and undergoing hypertrophy if not excessively shielded by internal fixation or protection from weight-bearing [47]. Up to 80% of structural vascularized bone grafts will show significant hypertrophy over time in response to stress loading by 24 months following reconstructive surgery [48, 49]. In many cases, however, fibular flaps require protection from mechanical loads during the hypertrophic process, particularly when placed to span a larger and stronger bone. Instead, the mechanical load should be gradually increased over time to promote bone remodeling and hypertrophy and minimize the risk of early stress fractures.

Indications / Contraindications

Autogenous free tissue transfers have become a relatively common technique for the reconstruction of complex bone and soft tissue defects. The use of these more complex reconstructive options should be considered only when simpler reconstructive methods have failed or are likely to fail. Generally, it is reasonable to consider a vascularized bone graft as a reconstructive option for bone defects larger than 6-8cm due to limb-sparing tumor surgery, but also due to traumatic loss, infection, or congenital pseudarthrosis [50-52]. Other indications include; a bone defect of smaller size which has failed to heal with non-vascularized bone grafts, a previously infected non-union with a segmental defect, a non-union with or without a defect due to radionecrosis, spinal and sacral defects, failed primary reconstruction, pathologic fractures due to osteonecrosis, or reconstructions which are likely to result in "biologic failure" [33, 34, 52, 53] [54]. The biologic failure can be caused by a damaged or poor soft tissue envelope, infection, previous irradiation, scar formation, and vascular injury. The position and fixation of the fibula in which the fibula is placed depends upon the location of the recipient site. In large diameter bones, the fibula is often combined with a large allograft, placed either within the medullary canal or as an onlay spanning the defect. Cases of persistent bony non-union, radionecrosis, and pathologic fractures can be treated by the onlay technique. Vascularized grafts may also be considered after failed attempts to obtain union by conventional techniques^{55, 56}. Relative contraindications for harvesting a free fibula graft are those patients for whom the harvest would cause insufficient lower extremity blood flow.

Pre-operative patient evaluation

A successful free vascularized bone transfer is depending upon careful preoperative planning and complete evaluation of the donor and recipient site. The characteristics of the recipient's site must be evaluated for its bony, soft tissue, and vascular anatomy to ensure appropriate graft selection [42, 57]. Free vascularized transfer of the fibula requires systematic evaluation of the donor site including the bony and vascular anatomy variations that may compromise graft harvest [58]. The evaluation includes a radiograph to rule-out previous fractures, and vascular examination with Doppler ultrasound is vital [42]. Pre-operative angiography should be considered routine in trauma cases, or if a preoperative Doppler exam suggests a vascular abnormality. In both trauma and post-irradiation cases, the extent of soft tissue damage may extend well above and below the defect. In such cases, the anastomosis should be planned away from the zone of injury. For this reason, it is sometimes preferable to perform a distal anastomosis instead of a proximal anastomosis. If osteomyelitis is present, one must make sure extensive debridement of all devitalized bone has been performed and adequate soft tissue coverage is present before vascularized bone grafting is considered [59, 60].

The technique of free fibula harvest

Anatomy

Like other long bones, the fibula shaft receives its blood supply through a combination of endosteal and periosteal vessels. Approximately 70% of the cortical blood supply derives from the endosteal vasculature. The remaining 30% of the cortex receives its blood supply through a transverse circulation from the periosteum [44, 61, 62]. The endosteal centrifugal blood supply derives from the nutrient artery branch of the peroneal artery entering the fibula in the middle third of the diaphysis through the nutrient foramen. The nutrient artery of the fibula arises 6-14cm from the peroneal artery origin [42, 58]. The periosteal blood supply derives from small branches of the peroneal and anterior tibial arteries in the middle third of the diaphysis [42]. The peroneal artery branches approximately 3 cm distal to the origin of the anterior tibial artery. The artery typically penetrates the soleus muscle close to the lateral intermuscular septum and continues distally in the leg parallel to the fibula, running in between the posterior tibialis and flexor hallucis longus muscle. It is accompanied by two venae comitantes on both sides of the artery. The peroneal pedicle has a length of 6-8 cm with an arterial diameter of 1.5-3.0 mm. The free fibula flap can be harvested with a skin paddle up to 10-20 cm. The skin paddle is vascularized by a series of fasciocutaneous or myocutaneous perforators who arise from the peroneal artery. The perforating vessels in the mid-fibula area are often found coursing through the flexor hallucis longus and peroneus longus muscles. In the distal fibula the perforating vessels are found between the soleus and peroneus brevis muscles. The exact location of the perforators can be determined by the use of Doppler ultrasound, thus skin paddle can be designed around these perforators. Due to the myocutaneous course of the perforating vessels, one could design composite grafts including bone, skin, and muscle. The flexor hallucis longus may be used and provides enough coverage for the distal fibular segment. A larger muscle sufficient to obliterate dead space or provide coverage may be needed. The lateral part of the soleus muscle can be used for this reason without loss of function. This can be accomplished by sparing the soleus muscle branches when visualized during the exposure of the peroneus pedicle [60].

The blood supply of the epiphysis becomes more important when longitudinal growth in children is necessary. The optimal vascular pedicle to include the proximal epiphysis of the fibula has never been agreed on. The proximal fibular epiphysis receives its vascular supply through an arcade of vessels derived from the lateral inferior genicular artery and the anterior tibial artery. This arcade is formed superiorly by branches of the inferior lateral genicular artery, and inferiorly by branches of the anterior tibial artery, the most important branches are the first and second recurrent epiphyseal arteries [63]. Raising a free fibula flap including the proximal epiphysis demands a different approach. When a long segment is harvested including the epiphysis it preferably requires a double pedicle including the anterior tibial artery or inferior genicular artery along with the peroneal artery for diaphyseal blood flow. One could dispute using either the branches of the anterior tibial artery or from the lateral inferior genicular artery. One study used only one pedicle (the anterior tibial artery) when raising a fibular flap including the growth plate with good results [64].

Donor site: free fibula harvest by a lateral approach [42, 57]

After induction of general anesthesia, intubation, and monitoring of the patient. The patient positioning should be supine with a bolster under the ipsilateral buttock, or in the lateral decubitus position on the operation, table depending upon optimal recipient site access. Thereafter, the entire fibula should be outlined with a marker (Fig. 1.1). The fibula graft should always include the middle third to include the nutrient artery. In the case of an osteocutaneous flap, the skin paddle is designed and outlined around identified perforators (Fig. 2). Depending on surgeons preference, a pneumatic tourniquet can be used after limb exsanguination. A longitudinal incision is made directly over the outlined fibula extending 5-6 cm above and below the required length of the fibula. In the case of an osteocutaneous flap, this incision is made through the *anterior* border of the skin flap, protecting the identified perforators posteriorly. Dissection through subcutaneous tissues will expose the underlying muscles. Distally, the broad peroneus longus tendon serves to identify the lateral compartment, centered on its muscle belly. A fat stripe is visualized posterior to the muscle belly (Fig. 1.2). The identification of this fat stripe, characterizes the anatomic interval between the posterior (soleus) muscle compartment and the lateral muscle compartment (peroneus longus and brevis). This anatomic interval or septum is a key landmark for further dissection of the fibula.

Once the muscle interval is identified, a limited posterior dissection is next performed in the proximal third of the fibula, to identify and protect the peroneal vascular pedicle. As peroneal cutaneous perforators generally lie more distally, they should not be an issue in this dissection. The posterior muscles are retracted posteriorly beginning at the fat stripe. Gentle probing will demonstrate the soleus origin firmly attached to the lateral and posterior aspect of the proximal fibula. It must be carefully elevated from the bone. (Fig 1.3). During this dissection, one or more vascular branches entering the deep (anterior) surface of the soleus are visualized, arising from the peroneal vessels. As the interval is developed, the proximal border of the flexor hallucis longus (FHL) muscle is visualized, covering the posterior surface of the fibular diaphysis. This is confirmed by visualizing the peroneal vessels passing distally deep (anterior) to the proximal edge of the FHL, as visualized once the soleus is released. Visualization of the vessel and FHL

proximally is important to prevent inadvertent dissection anterior to the FHL and potential injury to the vessel more distally. (Fig 1.4). If a composite osteomuscular flap (including soleus) is planned, the peroneal muscular branches to the soleus should be preserved, otherwise, these can be ligated and divided. The initial posterior dissection is completed by carefully elevating the peroneal vessels from the fibula to prevent injury when the proximal osteotomy is performed.

Next, the anterior dissection is performed. The dissection begins proximally and subperiosteally to first identify the peroneal nerve. Dissection directly on the bone surface is critical to protect the peroneal nerve at the level of the fibular neck. As the lateral compartment muscles are elevated from bone, the common peroneal nerve is visualized on the deep surface of the elevated muscles (Fig. 1.5). Its division into superficial (lateral compartment location) and deep (anterior compartment location) branches are next demonstrated. Once the nerve is visualized, the remainder of the dissection is extraperiosteal to preserve the periosteal blood supply of the bone.

Progressing distally, the lateral compartment muscles are elevated sharply, leaving wisps of muscle on the bone surface. Preserving a 'cuff' of muscle, as sometimes described, is erroneous, risking injury to both peroneal nerve branches and the tibialis anterior vessels. Besides, this potentially necrotic muscle cuff may also block angiogenesis to the periosteal surfaces from adjacent soft tissue.

Next, the anterior compartment musculature is elevated from the bone. Proximally, the deep peroneal nerve is identified. Once visualized, it is easily protected and proximal-to-distal extra-periosteal elevation of the anterior compartment is performed. The anterior dissection at this point stops with the visualization of the interosseous membrane and tibialis anterior vessels.

Next, the fibula may be divided. We prefer to take more fibula than recipient-site measurements suggest to be necessary. Loss of the fibular diaphysis is well tolerated in the donor leg. Importantly, the optimal positioning of the fibula in the recipient site often requires adjustment, to maximize pedicle length and position for local vascular anatomy, and given the variability in nutrient artery location in the fibula. Harvest of a short fibular segment initially risks the potential loss of its endosteal blood supply.

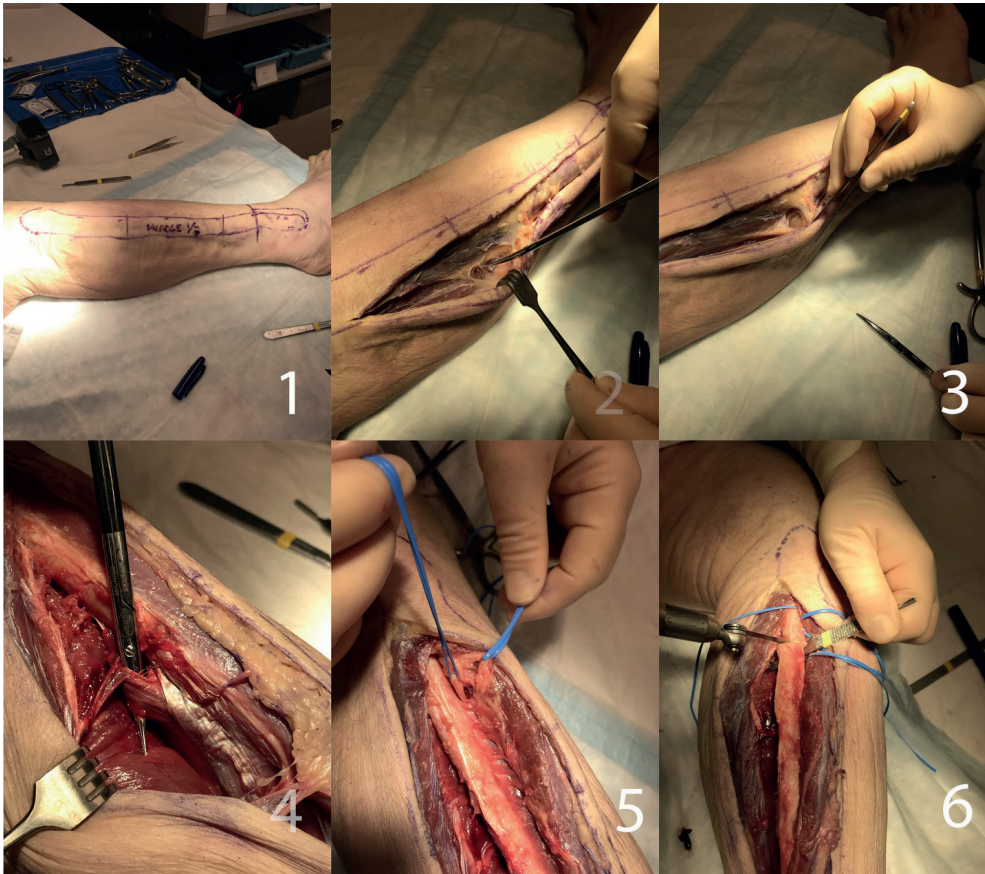


Figure 1: pre-operative outline of the fibula by palpation, the to be harvested segment should include the middle 1/3 part of the fibula to include the nutrient artery (1), a longitudinal skin incision is made and the fat stripe between the peroneus and soleus muscle identified (2), Blund dissection of this interval is made beginning in the middle 1/3 of the bone (3), dissection is continued proximately and the soleus detached of its origin and the peroneal vessels identified (4), the peroneal nerve is then identified at the neck of the fibula and the lateral compartment muscles elevated (5), a subperiosteal dissection is carried out proximately and the proximal osteotomy is made (preferably with a Gigli saw) while protecting the neurovascular structures (6).

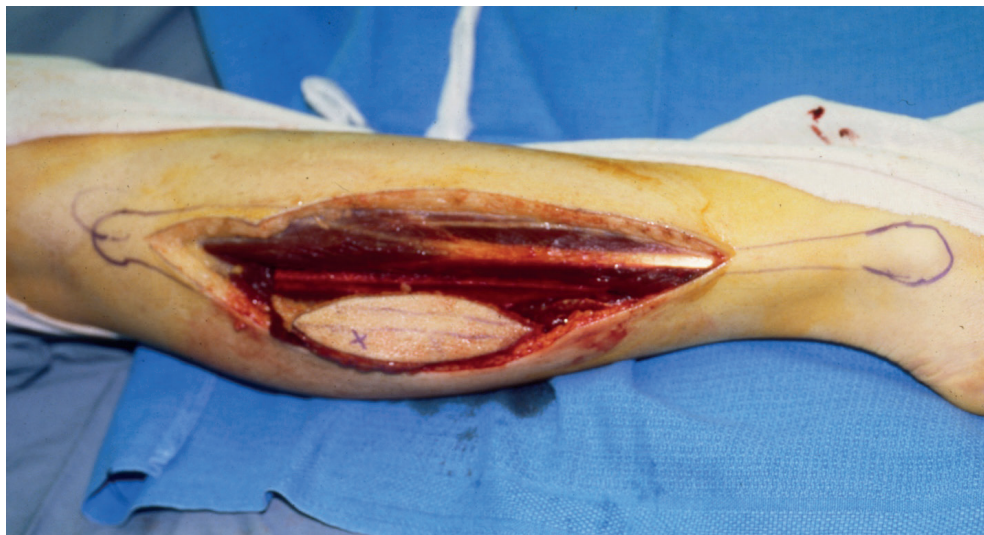


Figure 2: If desired a skin paddle can be designed based on perforator vessels of the peroneal vessels, by using a Doppler ultrasound. Here we demonstrate an example of dissection of an osteocutaneous free fibular flap.

While carefully protecting the previously mobilized neurovascular structures with gentle retraction, the proximal osteotomy is to be made with a Gigli saw (Fig. 1.6). Next, the distal cut made, maintaining 7-8 cm of the distal fibula at the ankle, to prevent valgus ankle instability (Fig 3.1). In children, a distal tibiofibular syndesmotic fusion must be performed to safely avoid valgus deformity regardless of the length of the remaining fibula. Once both osteotomies are made, the fibular flap mobility facilitates the remainder of the dissection.

Next, the fibular harvest is completed by distal-to-proximal dissection. The interosseous membrane is sharply divided, and the fibula gently pulled laterally (Fig. 3.2). The tibialis posterior muscle lies directly behind the interosseous membrane, covering the peroneal vessels from an anterior view. At the distal end of the fibula, dissection between the tibialis posterior and flexor hallucis longus permits visualization of the peroneal vessels distal to the fibular bone flap (Fig. 3.3). The vessels are doubly-ligated at this level. Leaving one suture long facilitates subsequent exposure of the vessels, now covered by the tibialis posterior muscle by the use of gentle longitudinal tension. Tension stabilizes the vessels as the tibialis posterior muscle is divided. The plane of dissection is directly anterior to the peroneal bundle. Meticulous vessel exposure begins distally, dividing the tibialis posterior muscle into short segments and a layered fashion. This permits identifying and ligating multiple peroneal muscular perforators as they are encountered (Fig. 3.4). If an osteocutaneous fibular flap is to be used, division of the tibialis posterior must pause and the cutaneous perforators dissected. They may lie either within the muscle or in the lateral intermuscular septum. The posterior border of the skin flap is made at this point, and the perforators followed to the peroneal vessels. Dissection is continued proximally until the tibialis posterior is completely released from the fibula, leaving only the vascular pedicle and flexor hallucis longus muscle attached.

Finally, the flexor hallucis longus muscle is dissected from the fibular segment. Again, dissection progresses from distal, meticulously identifying and ligating or bipolar-cauterizing small muscular perforators. The dissection is performed directly on the vessel surface rather than at the fibula itself. This results in a small strip of remaining muscle lying on the fibula and next to the peroneal vessels, protecting the bone blood supply. At this point, the fibular segment is fully isolated on the peroneal artery and associated veins. The peroneal vessels should be fully mobilized up to the point where they join the posterior tibial vessels (Fig. 3.5). Any remaining muscular branches should be ligated and divided. If a tourniquet is used during the harvest procedure, release the tourniquet for 5-10 min before the final cutting of the pedicle to perfuse the bone while getting hemostasis of the leg. Depending on surgeons preference, two suction drains can be placed - one in between the flexor hallucis longus and soleus, the other subcutaneously. The flexor hallucis longus is loosely repaired to the peroneal muscle with a running absorbable suture and the skin closed in layers (Fig. 3.6).

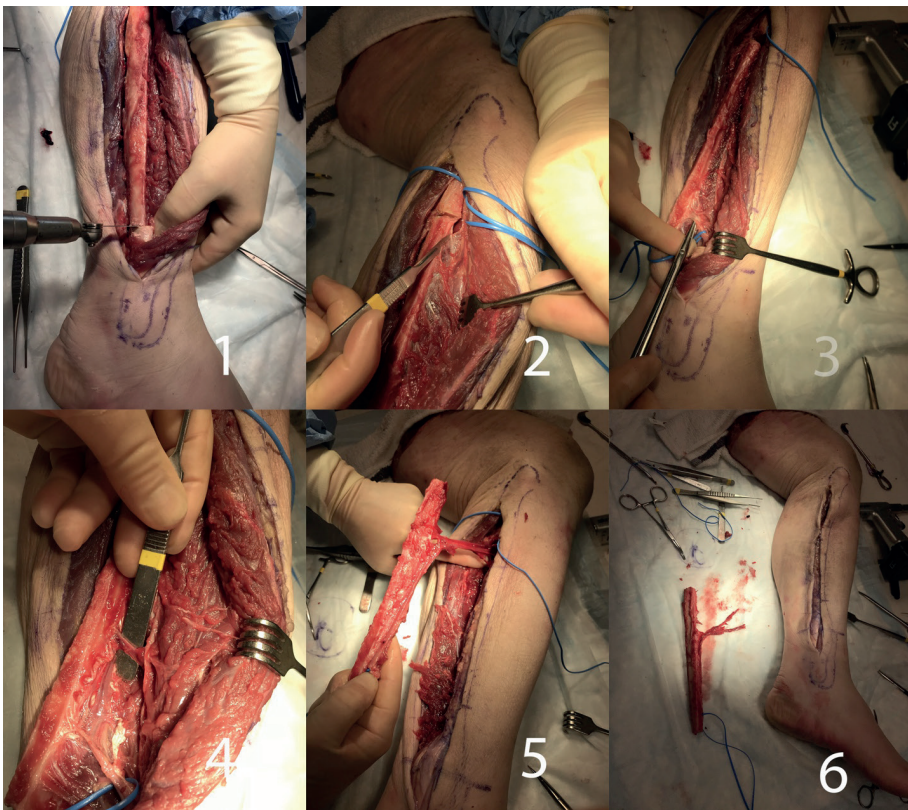


Figure 3: The distal osteotomy should be made 7-8 cm above the ankle joint (1), the fibula can now be rotated laterally and the interosseous membrane sharply divided (2), the peroneal vessels are identified and ligated at the distal end of the osteotomy leaving a silk tie long at the proximal stump will facilitate dissection of the vessel from distal to proximal (3), the tibialis posterior muscle is divided just above the peroneal vessels and muscular branches ligated (4), the flexor hallucis longus is then divided of the bone, now the fibula is isolated on the peroneal vessels (5), before the final ligation and cutting of the pedicle vessels, release the tourniquet for 5-10 min to perfuse the bone while getting hemostasis of the leg (6).

Surgical technique recipient

Tibia

Each recipient site represents different challenges regarding vascular access, fixation, and post-operative management. For tibial defects, the ipsilateral fibula may be used as a pedicled autograft. This significantly reduces operative time, as there is no need for a microsurgical anastomosis [65]. When missing, fractured, or is surrounded by damaged and heavily scarred soft tissue, the use of the contralateral fibula as a free flap is required.

Initial vessel dissection in the recipient can be performed either before the fibula harvest or at the same time if two surgical teams are used. Pre-operative planning is most crucial when a free flap is used. Determine where the best suitable recipient vessels are located, how the fibula is going to be orientated (orthograde/antegrade), what type of fixation is needed, and if an additional massive allograft is needed. A pre-operative angiogram can therefore especially useful to determine the right recipient vessels and location. Depending on recipient-site vessel availability, the free fibular flap may be oriented in either orthograde (for anastomosis to the anterior tibial artery) or retrograde fashion (for anastomosis to the posterior tibial artery) [57].

For metaphyseal and diaphyseal reconstruction the fibula can be best placed intramedullary. If it does not fit well inside the tibia, the best alternative is to place an allograft matched to the defect (fixed with an IM-nail) and an onlay fibula spanning the defect secured with lag screws for compression. In case of using a fibula alone, one could center the fibula and ream the medullary canal of the tibia to fit the fibula.

Stable internal fixation is crucial to achieve union at both docking sites. Stability can be provided by internal fixation spanning the defect (locked IM nails or spanning locking plates and screws). The type of fixation is depending on the position of the fibula with or without an allograft. Fixation of the fibula only with small fragment plates and screws above and below or with external fixation result in higher complication rates [34, 42, 66].

When bony fixation is achieved vascular repair of the free fibula can be performed by a microanastomosis. Preferably end-to-side anastomoses are used when possible to preserve distal blood supply. Post-operatively, immobilization and non-weight bearing are advised until there is radiographic evidence of healing. Persistent non-union at the docking site 6 months after the initial transfer should be treated with additional cancellous bone graft. After the union of both docking sites, mobilization and weight-bearing can be carefully initiated. Partial or protected weight bearing is advised until radiographic evidence of fibular hypertrophy (Fig. 4D) [57]. In children, non-weight bearing is required following syndesmotic fusion. When an osteocutaneous flap is taken, direct closure of the donor site is ill-advised, due to the risk of a compartment syndrome. A split-thickness skin graft should be used to complete skin closure.

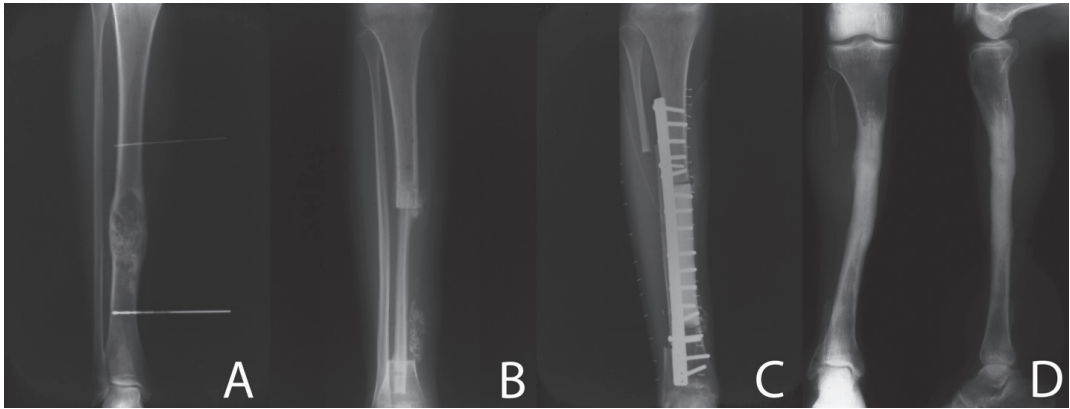


Figure 4: pre-operative X-ray of a bone tumor in the tibia while planning the margins of the resection (A), post-operative X-ray a large segmental defect of the tibia treated by a free vascularized contra-lateral fibula (B), large defect of the tibia reconstructed with a large allograft to obtain union an ipsilateral pedicled fibula was used as an onlay graft (C), conventional x-rays demonstrating hypertrophy of a pedicled ipsilateral fibula used for the reconstruction of a large segmental defect in the tibia.

Femur

Femoral reconstructions oppose a considerably more challenging problem, compared to the tibia. The femur presents an array of technical difficulties particular to this bone because of greater difficulties with bony stabilization and vascular access. The greatest challenge in this type of reconstruction is to achieve a strong and stable situation with rigid internal fixation. Strong muscle forces across the bone can result in instability and fractures resulting in poorer outcomes. Appropriate pre-operative planning in these cases is essential so initial vessel dissection, flap harvest, bony fixation, and anastomosis can be performed in an orderly and efficient way. A multi-disciplinary approach is therefore advised.

For femoral reconstruction, the ipsilateral fibula is the preferred donor site, unless other contraindications exist. In most cases two incisions can be used, one medially for vascular access and one laterally for bony access. Due to the greater cross-sectional size of the femoral shaft, the fibula can be placed within the intramedullary canal with a spanning plate and screws (Fig. 5), alongside an IM nail, with direct end-to-end contact, as an onlay graft spanning a defect reconstructed with a structural allograft and an IM nail.

In the femur, the fibula is prone to a stress fracture if not protected from direct mechanical loads. Therefore, the free intramedullary placed fibula can be augmented with a size-matched structural allograft and stabilized with a spanning plate^[67]. Eccentrically placed fibulas are for the same reason often supported by a locked IM nail. The third option for fibular placement in femoral reconstruction is the onlay technique where the fibula is placed on top of a cortical allograft^[57]. All methods call for rigid internal fixation. The final option for femoral reconstruction is a double-barrel flap. With this technique, the free fibula is osteotomized at its midpoint without dividing the vascular pedicle after harvest. This produces two vascularized bone grafts that require only one set of vascular anastomosis and provides the double cross-sectional area of a single fibula transfer when placed in a single bone^[60].

For mid-femoral defects, the superficial femoral artery and vein can be used as recipient vessels for most mid femoral defects with side-to-end anastomosis for the artery and end-to-end venous anastomosis. In proximal defects, the lateral femoral circumflex vessels can be used. For very distal femoral defects the popliteal vessels can be used in an end-to-side fashion, through a posterior approach. If possible, the anastomosis is best made to the superficial femoral artery in distal defects since the approach to the popliteal vessels often requires the intra-operative turning of the patient. Secondly, the anastomosis will be in an area where joint mobility can play a factor in blood flow disturbance^[57].

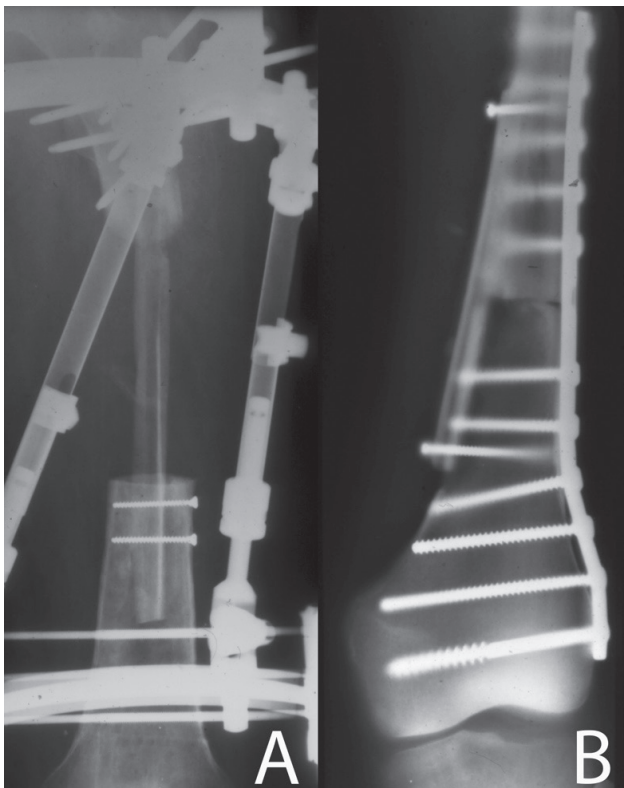


Figure 5: post-operative X-ray of a large segmental defect reconstructed with a free fibula graft and fixed with external fixation (A), Post-operative X-ray of a large defect of the femur reconstructed with an allograft combined with a free fibula graft as onlay graft to increase healing (B).

Flap monitoring

Thrombosis of the vascular pedicle results in impaired outcomes since the autograft has to incorporate by means similar to those of conventional non-vascularized allografts. They can result in late stress fractures and eventual resorption of the bone graft. The monitoring of a vascularized bone graft in the immediate post-operative period is therefore desirable ^[60]. Graft viability can be assessed by a variety of methods. Continuous monitoring of the flap is possible by the use of implantable or surface Doppler ultrasound. Although measuring the blood flow in an embedded free flap is difficult, special implantable probes have been used effectively for at least 48h postoperatively. The use of implantable devices carries the risk of thrombosis, spasm, and dislocation of the microanastomosis with subsequent hemorrhage ^[68, 69]. The most feasible and easily interpreted continuous monitor is a fasciocutaneous “buoy” flap. Direct monitoring of the cutaneous island allows immediate return to the operating room if loosening of the dressing, altering limb position, or anticoagulants do not reverse the observed changes. In lower extremity reconstruction, especially in femoral reconstructions, a buoy flap is often impractical since due to the depth of the fibula placement. Osteocutaneous or osteomuscular flaps can be harvested when additional soft-tissue coverage is needed. Intermittent monitoring methods of the fibula flap are more practical and include; angiography, radiographic monitoring of healing and hypertrophy, bone biopsy, early 99Tc bone scans, Doppler ultrasound or SPECT scans. All of these methods are a single time point assessment of viability and do not allow immediate action in case of impaired vascularity. Intermittent monitoring with bone scans correlates with vascular patency when completed before the end of the first week ^[71].

Outcomes

Union

The most common indication for the use of vascularized bone transfers is the need for reconstruction of a skeletal defect after radical resection of malignant bone tumor or metastasis (Fig 4). The primary union rate in this group of patients is 67-84%, after secondary bone grafting the rate of union is 81-92% ^[23, 72]. After tumor resection, infection is the most common indication for vascularized bone reconstruction. Patients who have segmental bone loss due to infection, have the lowest rate of union (47-77%) and, if unsuccessful, may result in amputation. Patients who do not have an infection have the best chance of healing with a primary union rate of 70-77% and an overall union rate of 92-95% after secondary procedures to achieve union ^[23, 73].

Functional outcome (MSTS)

The Musculoskeletal Tumor Society scoring system (MSTS) is a disease-specific measure for the physical and mental health outcome after limb-sparing surgery in the lower extremity. The MSTS scoring system consists of six domains, scored on a 0 to 5 scale, and transformed into an overall score ranging from 0 to 100 percent score (or 0-30 points) with a higher score indicating better function. A review of 35 vascularized free fibula grafts for the reconstruction of lower extremity defects after malignant tumor resection demonstrated a mean score of 88 percent^[74]. When vascularized fibular grafts are used in lower extremity reconstruction and protected with a cryopreserved allograft the mean MSTS reaches 87.8 percent found in a systematic review of 123 patients^[75].

Factors that influence the outcome

Multiple factors play an important role to achieve final bone union, reduce complications and re-operations. A review of vascularized bone grafts for both upper and lower extremity reconstruction demonstrated that rigid internal fixation and use of additional bone graft results in significantly better union scores^[23]. Significant risk factors found in literature for non-union are the use of Tobacco, post-operative chemo/radiotherapy, and reconstruction performed for osteomyelitis^[1, 66, 74]. A trend toward more non-union is found for those patients who have diabetes and patients with an age above 40 years^[23].

Complications

Donor site complications

Complications related to the donor site reported in literature for free fibula harvest include (chronic) pain, altered gait, weakness, contracture of the flexor hallucis longus, compartment syndrome, peroneal nerve palsy, valgus deformity, arterial insufficiency, and spontaneous fracture of the ipsilateral tibia. Donor site complications are generally fairly minimal and a review of 132 free fibula grafts demonstrated donor site complications in 8 percent of the patients^[1]. Although a compartment syndrome after fibula harvest is rare, a split skin graft must be used to close the wound when an osteocutaneous fibula is harvested.^[60] An extensive review of 247 lower limbs from which a free fibula was harvested was evaluated specifically on donor site morbidity. This review showed mild weakness in 10 percent of the patients and sensory deficit in 5-12 percent of the patients. Chronic pain was present in 9 percent of the patients^[76]. Although donor site complications are mostly transient and generally an acceptable trade for successful limb salvage. A known complication in children is a valgus deformity of the ankle after fibula harvest. To prevent this complication, fusion of the distal tibiofibular joint just proximal to the physis must be performed^[77]. Occasional flexor hallucis contractures may require tendon lengthening or tenotomy.

Recipient site complications

Thrombosis

Complications related to thrombosis are compartment syndrome at the recipient site, late stress fracture, and non-union. Due to thrombosis, blood flow, bone formation, and osteocyte counts are reduced. This can ultimately lead to late stress fractures and non-union. If left untreated, thrombosed vascularized bone grafts are significantly less likely to heal compared to conventional non-vascularized grafts [78]. If thrombosis occurs, the surrounding cuff of necrotic soft tissue may likely impede neovascularization. Thus, the process of creeping substitution of necrotic bone is less extensive in these failed vascularized bone grafts.

Delayed union

The need for secondary bone grafting is necessary for a substantial number of patients with delayed union. Primary healing rates have been reported to be around 68% of the vascularized fibular grafts, with poorer results depending on some recipient site locations and in patients with osteomyelitis [1]. Those patients who do not heal primarily may benefit from a secondary intervention with autogenous corticocancellous bone grafting at the non-united junction. Forty-five percent (45%) of the total reoperations after the vascularized fibular transfer are the result of delayed union treated by supplemental autogenous bone grafting [74]. The total healing rate after secondary intervention reaches 82-88% found in literature [1, 49].

Stress Fracture

Stress fractures of vascularized bone graft are not uncommon, they are particularly prevalent in lower extremity reconstructions. In literature, stress fractures occur in the lower extremity in 8-27 percent of the patients [1, 74, 79, 80]. Stress fractures can be divided into two groups: early stress fractures and late stress fractures. Early stress fractures are the result of insufficient fixation or protection from mechanical loads since the fibular graft has had insufficient time to undergo hypertrophy (Fig. 4D). Rigid internal fixation with spanning plates or intramedullary fixation of fibular grafts may result in a lower incidence of fracture [81]. If the graft has adequate vascularity, the healing of undisplaced stress fractures may occur with immobilization (Fig. 6). Whereas displaced fractures require internal or external fixation with or without bone grafting [1, 79]. Late stress fractures are relatively uncommon and are associated with impaired vascularity. Vascularized fibular grafts should be protected from fatigue fracture during the first year, allowing a gradual increase in mechanical load, which enables remodeling and hypertrophy[48].



Figure 6: A stress fractured occurred after the reconstruction of a large tibia defect with a free vascularized fibula. This radiograph shows the normal healing process of the fracture due to the maintained tissue viability of the vascularized fibula flap.

Infection

Osteomyelitis is one of the second most frequent indications for vascularized bone transfer [1]. The outcome of these patients is less satisfactory. Experience with a series of 60 patients who had a vascularized bone transfer for osteomyelitis demonstrated an increased potential for several complications. These complications include reactivation of the infection (18%), occasionally leading to amputation(17%), and non-union(6%) [1]. To prevent reactivation of the infection vascularized bone transfer must be delayed until sepsis of the recipient site is inactive. Criteria used to for determining inactivity of the recipient site are negative bacterial cultures, absence of sinus tracts, negative C-reactive protein testing, sedimentation of less than 15mm/hr, for at least 1 month after the last episode of infection [59]. Infections also occur in patients that did not have an infection at the site of the defect. The incidence of postoperative deep infection after vascularized bone transfer alone in this patient group has been reported to be 10% [1].

Other complications

Other complications include limb-length discrepancy, mal-union, and tumor recurrence. As many primary bone tumors affect children and adolescents, development and growth need to be taken into account and might lead to a limb-length discrepancy. Therefore, methods chosen must enable bone lengthening, whether by replacement of missing physis or some other method permitting reconstruction of limb length discrepancy [4, 82]. Another method of limiting limb length discrepancy involves epiphysiodesis of the contralateral limb. In tumor cases, local recurrence of the tumor is possible due to incomplete tumor resection or graft metastasis. Post-operative chemotherapy or radiation are frequently used methods to reduce the risk of local recurrence and distal metastasis. With increasing survival rates after limb-sparing surgery, a vascularized bone transfer can be safely performed after resection of primary malignant bone tumors [66, 82]. Mal-union is a relatively uncommon complication but might occur due to excessive ambulation or instability of the reconstruction, while the initial alignment had been satisfactory [72].

Future considerations

The ability to safely use vascularized composite allotransplantation (VCA) would be a significant development in the reconstruction of large segmental bone defects. Allotransplantation of living bone or joint segments is a form of vascularized composite allotransplantation such a face, abdominal wall, and hand transplantation. Living bone allotransplants combine the ability to match defect size and shape, similar to cryopreserved banked bone, with the enhanced healing and remodeling potential of living bone [34, 47, 83-86]. Bone and joint VCA's have been performed only sporadically in a clinical setting, due in part to the need for long-term immunosuppression. The risks and expense of drug immunotherapy are a major obstacle to their use. The use of immunosuppressive regimens needed for VCA cannot be justified in patients with nonlethal conditions. Long-term immunosuppressive regimens may be circumvented by replacing the allogenic bone circulation with a neoangiogenic autogenous blood supply. The autogenous neo-angiogenic circulation is created by implanting an autogenous arteriovenous bundle into the intramedullary space of a vascularized bone allotransplant (Fig. 7). This method would require only short-term immunosuppression. This method has proved to have potential in experimental models in our laboratory.

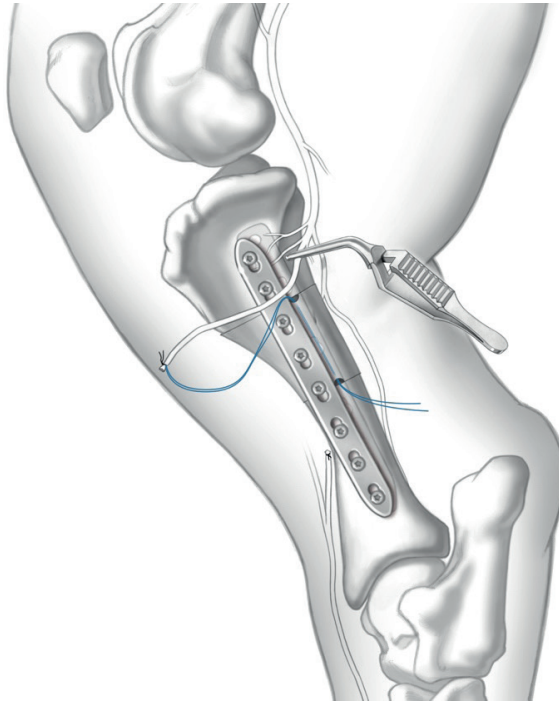


Figure 7: porcine hind-limb model demonstrating a vascularized tibia segment transplanted into a recipient, while simultaneously implanting an autogenous arteriovenous bundle into the intramedullary space. The arteriovenous bundle induces autogenous revascularisation within the allotransplant and maintains viability with only short-term immunosuppression.

Summary

Vascularized bone transfers have been used since the 19th century for the treatment of skeletal defects because of faster union rates, fewer fatigue fractures, rapid hypertrophy, and less resorption compared with non-vascularized bone transfer [49, 87]. Its structure and shape make it particularly useful for diaphyseal reconstruction; a straight segment of bone between 26 and 30 cm can be harvested, and stability can be obtained rigid internal fixation to the recipient's bone. For tumor indications, the use of microvascular reconstruction results does not result in an increased risk of local recurrence or death from metastatic disease. Primary union rates have been found in literature 67-84%. However, after secondary bone grafting the rate of union is 81-92% [23, 72]. Complication rates after vascularized bone grafting for reconstruction of a bone defect in the lower extremity are strongly dependent on location, and underlying reason of the defect. Patients who were treated with a vascularized bone graft for treatment of a large bone defect due to osteomyelitis have the highest risk for complications. Vascularized autografts are limited by the few available expendable donor sites, and by donor site morbidity. However, vascularized bone grafts currently remain the golden standard for the reconstruction of large segmental bone defects while complications are manageable.

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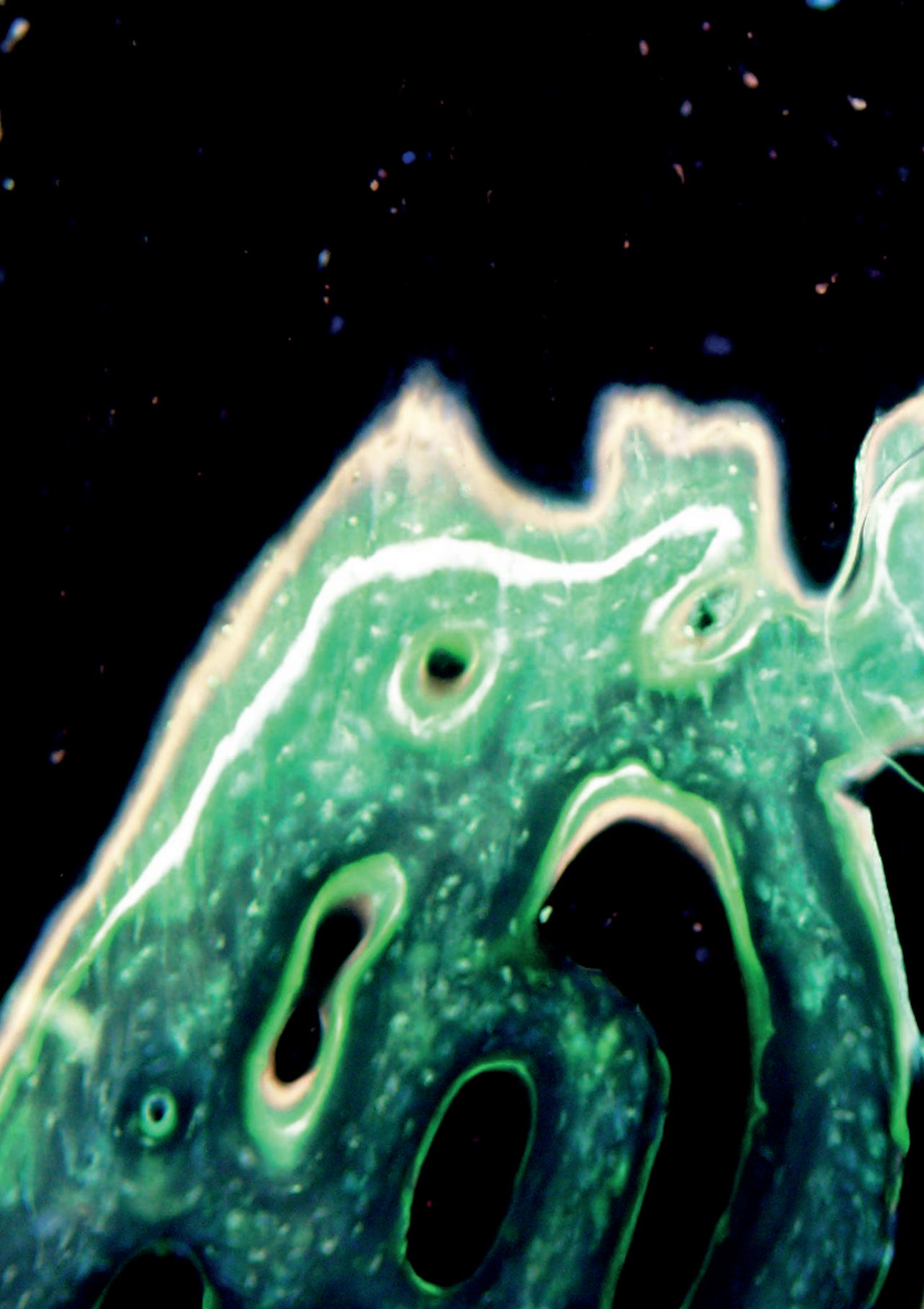
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3

CHAPTER

**Combined massive
allograft and intramedullary
vascularized fibula as the
primary reconstruction
method for segmental bone
loss in the lower extremity:
a systematic review and
meta-analysis of literature**

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Abstract

Background: Reconstruction of segmental bone loss due to malignancy, infection or trauma is a challenging issue for the reconstructive surgeon. The combination of a vascularized fibula flap with a cortical allograft provides a reliable reconstructive option in the lower extremity. In this systematic review of literature, we describe the outcome of this technique for the treatment of segmental bone loss.

Methods: A systematic review was performed on the use a combined massive allograft and intramedullary vascularized fibula as a reconstruction method for large bone defects. We used PubMed, EMBASE and Wiley Cochrane Library. No language restrictions were imposed.

Results: Seventeen clinical articles were included between 1997 and 2017, reporting 329 cases of lower extremity reconstructions. A meta-analysis was performed on primary union rates. Main outcome measures were primary union, complication rate, re-intervention rate, and function after reconstruction. All publications show relatively high complication (5.9-85.7%) and re-intervention rates (10-91.7%) with good primary union rates (66.7-100%) and functional outcome (MSTS-score: 24-29).

Conclusions: The combination of a massive allograft with intramedullary vascularized fibula provides a single step reconstruction method for large bone defects (<6 cm) in the lower extremity with good long-term outcomes.

Introduction

Segmental bone loss is a serious and challenging problem after limb saving oncological surgery, severe complex trauma, and infection [1]. Methods used to restore segmental bone loss include vascularized autogenous bone, the Masquelet technique, distraction osteogeneses, prosthetic replacement, re-implantation of autoclaved tumor bone and massive allografts used alone or in combination with a vascularized autograft [2-5]. The Masquelet technique [6] is a commonly used and well-known technique in trauma. This technique uses a combination of unvascularised autologous bone graft and an induced membrane in a specific time frame. The downside of this two-stage reconstruction method is the long time it takes before full weight bearing is allowed and its association with infection, resorption, and non-union [7]. Distraction osteogenesis is also commonly used in trauma cases but is associated with pin-tract infection, soft tissue problems, non-union at the docking site, and prolonged non-weight bearing [8, 9]. Prosthetic replacement is associated with failure rates ranging from 33% to 100%, requiring revision or amputation for mechanical failure and/or infection [10-12]. Massive allografts alone or re-implementation of autoclaved tumor bone are both relatively simple reconstruction methods. They provide a “like with like” tissue reconstruction with immediate strength and no donor site morbidity. However, these non-vascularized bone grafts have many disadvantages and are associated with complications as non-union, infection and graft fractures. This is thought to be due to the avascular status of the graft [7, 13-19]. The vascularized fibula flap provides a good option for the reconstruction of large segmental defects. Vascularized bone grafts contain an intrinsic blood flow, provide osteogenic factors to the reconstruction and are capable of osteogeneses. These factors result in faster union times, improved strength and healing [20]. The downside of the use of a free or pedicled vascularized fibula flap is the risk of flap failure and the need for a donor site with its associated morbidity. In addition, there is a possible mismatch in size and strength between the original bone and the fibular graft, due to a smaller diameter of the fibula. This mismatch may necessitate a long period of non-weight bearing and can lead to fractures of the fibular graft. A combination of a vascularized fibular graft with a massive allograft provides the benefits of both methods and is also known as the Capanna technique [21]. Immediate strength and bulk provided by the massive allograft and the potentials of a vascularized fibular graft for primary union, hypertrophy and resistance to infection [7, 13, 15-19, 22-30]. Current literature on the outcome of the Capanna technique is limited by mostly retrospective studies with small sample size and conflicting results. In order to better understand the existing data, there is a significant necessity to further evaluate this data. This systematic review focuses on union rate, complication rate, re-intervention rate, time to full weight bearing, and functional outcome after reconstruction with massive allograft combined with vascularized fibular inlay.

Materials and Methods

Literature search

A review protocol was developed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA)-statement. (www.prisma-statement.org). A comprehensive search was performed in the bibliographic databases PubMed, Embase.com, and Wiley Cochrane Library from inception up to April 21, 2017, in collaboration with a medical librarian. The following terms were used (including synonyms and closely related words) as index terms or free-text words: “Capanna*”, “Vascularized allograft*”, “Vascularized autograft*”, “Lower extremity*”. The search was performed without date, language or publication status restriction.

Selection of studies

Two independent reviewers (RH and MR) screened all articles on title and abstract. After this first screening on in- and exclusion criteria, the remaining articles were screened on full-text eligibility. In case of disagreement between the two reviewers, a third reviewer (HW) was consulted (Fig. 1). Because the original article describing the Capanna technique^[21] was not found in the databases we included the original article into our search.

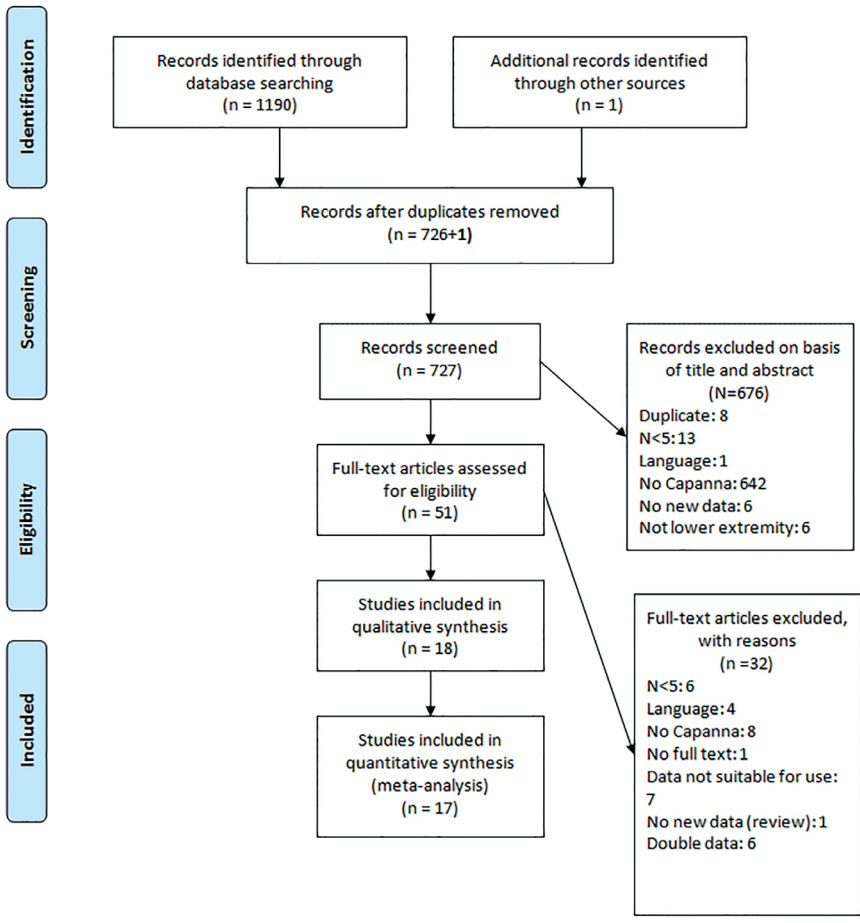


Figure 1: Flow diagram of study selection.

Inclusion criteria

In this systematic review, studies describing the use a massive allograft combined with vascularized fibula flap for the reconstruction of segmental bone defects were included. Randomized controlled trials, prospective and retro-prospective cohort studies, case-control studies and case-series published between January 1980 and April 2017 were included. The levels of evidence were scored with the use of the method described by the Oxford Centre for Evidence-Based Medicine 2011 [31].

Exclusion criteria

Upper extremity reconstructions were excluded. Case reports or case series describing fewer or equal to five cases were also excluded. We limited this study by language other than English or Dutch. No limitations for the follow-up period were made. When double data from the same author was suspected, we contacted the author. If the difference in the number of cases was fewer than five, the article with the smallest number was excluded. Animal studies and reviews were also excluded.

Data extraction

Data was independently extracted by two reviewers (RH and MR) on a preconceived data extraction form using Excel. The following data was extracted: first author, year of publication, level of evidence, blinding, population characteristics, underlying pathology, complications, union rates, graft characteristics, fixation method, immobilization method, time to full weight bearing (FWB), re-intervention, function, and amputation. Delayed union was defined as failure to reach bone union after within 6 months after reconstruction. Whereas non-union is defined as failure to reach bone union after 6 months without surgical intervention. When data or articles could not be provided by the library an attempt to contact the author was made. After independent data collection, the reviewers compared the extracted data.

Statistical analysis

Statistical analysis and graphic design were done in collaboration with the Department of Epidemiology and Biostatistics using MedCalc, version 17.8.2. A Meta-analysis was performed on 16 of the included studies for primary union rate. The other data could not be used for meta-analysis because of the heterogeneity of the data (Fig. 1).

Results

Study selection

A total of 1190 results were returned after a search in Pubmed, Embase, and Medline. The original articles (1989, 1991 and 1993) of the Capanna technique were not found by the performed search. One of the original Capanna articles was retrieved by the use of Google Scholar ^[21]. After removal of duplicates, screening on title and abstract and full-text assessment 17 articles were used for qualitative syntheses. Meta-analysis was performed on 16 included studies for primary union rate only. The other data could not be used for meta-analysis because of the heterogeneity of the data. A summary of our study selection is given in our flow diagram (Fig. 1).

Study characteristics

The included studies (n=17) were published between January 1997 and May 2017. The average follow-up was 58.5 months (15.0 -139.3). The included studies report their results of a massive allograft combined with a vascularized inlay for reconstruction of the lower extremity (femur + tibia) in 329 cases. The average age reported in all studies was 16.0 years (3-54). All studies presented results of reconstructions after oncologic resection, except for the study of Venkatramani et al.

[7]. This study described results in a series of trauma cases (n=6). The evidence in all studies was level IV, except for the study of Manfrini et al. [25] which was a case-control (level IIIb). This case-control study compared the combination of a massive allograft and centralized (pedicled) fibula flap, with use of a massive allograft and a free vascularized fibula flap. In our review, we included both techniques. A summary of all the study characteristics is shown in Table 1.

Table 1: Study characteristics

Author	Study design	Operation period	N	Gender	Age in years (range)	Mean Follow-up (months)	Pathology
Yang et al. ,2010	Case series	2001-2005	17	7F/10M	18,4 (6-34)	20.2	oncologic
Weichman et al. ,2015	Case series	2003-2011	12	7F/5M	15,8 (3-49)	41.4	oncologic
Venkatramani et al. ,2015	Case series	2012- 2013	6	6M	33 (18-49)	15	trauma
Rabitsch et al. , 2013	Case series	unknown	12	8F/4M	17,8 (11-31)	38.7	oncologic
Ozaki et al. ,1997	Case series	1991-1993	12	5F/7M	14 (7-54)	32	oncologic
Moran et al. ,2006	Case series	1997-2002	7	2F/5M	10,5 (5-18)	52	oncologic
Manfrini et al. , 1999	Case series	1989-1996	10	unknown	10,8 (7-12)	64	oncologic
Manfrini et al. , 2017	Case control	1994-2013	47	14F/33M	14,6 (6-38)	84	oncologic
Li et al. ,2010	Case series	2003-2008	11	6F/5M	18,5 (11-32)	34.1	oncologic
Li et al. , 2015	Case series	200-2011	11	5F/6M	12 (9-16)	48	oncologic
Jager et al. ,2010	Case series	2003-2010	7	1F/6M	16,5 (8,5-17)	44.1	oncologic
Innocenti et al. ,2009	Case series	1988 -2006	21	8F/13M	18,1 (5-52)	139.3	oncologic
Houdek et al. ,2016	Case series	1997-2012	18	9F/9M	11 (5-18)	96	oncologic
Halim et al. ,2015	Case series	1999-2012	10	unknown	19,8 (5-44)	63	oncologic
Erol et al. ,2015	Case series	2005-2012	7	3F/4M	10,4 (4-15)	38,9	oncologic
Ceruso et al. ,2008	Case series	1994-2007	31	unknown	14 (4-31)	75	oncologic
Capanna et al. ,2007	Case series	1988-2002	90	unmown	unknown	108	oncologic

Reconstruction methods

A total of 236 cases of tibial reconstruction and 93 femoral reconstructions are published in the included 17 articles. Six articles presented only tibial reconstructions and one study presented only femoral reconstructions. In 52 cases a pedicled fibula graft was used and in 277 cases a free vascularized fibular graft was used, both in combination with a massive allograft. In all studies plate fixation, interfragmentary screws, K-wires or a combination was used for fixation. Both minimal fixation methods and extensive internal fixation methods have been reported. Minimal fixation methods such as small fragment screws combined with a plate and more ridged fixation methods as large plates spanning the entire defect and even dual-plating. See Table 2 for a detailed summary of the used reconstruction methods and flap monitoring details per study.

CHAPTER 3

Table 2: Reconstruction method by study

First Author	Year	Patients	Site of reconstruction	Free/ Pedicled	Allograft	Fixation method	Flap control (N)
Yang et al.	2010	17	9 Tibia / 8 Femora	Free	Massive allograft	Plate & screws	skin paddle
Weichman et al.	2015	12	4 Tibia / 8 Femora	Free	Massive allograft	Plate & screws + K-wire	implantable Doppler/skin paddle
Venkatramani et al.	2015	6	6 femora	Free	Femoral allograft	Plate & screws	skin paddle
Rabitsch et al.	2013	12	5 Tibia / 7 Femora	Free	Massive allograft	Plate& screws	unknown
Ozaki et al.	1997	12	12 Tibia	Pedicled	Massive allograft	Plate & screws, K-wire, screws	unknown
Moran et al.	2006	7	3 Tibia / 4 Femora	Free	Massive allograft	Plate & screws	Radiological with bone scan day 10
Manfrini et al.	1999	10	10 Tibia	Free	massive tibia allograft	Small fragment screws proximal, small plate distal	radiological follow-up
Manfrini et al.	2017	47	47 Tibia	Free (22)/ Pedicled (25)	Massive allograft	Plate & screws	skin paddle, radiological follow-up
Li et al.	2010	11	6 Tibia / 5 Femora	Free (7)/ Pedicled (4)	Massive allograft	Plate & screws	radiological follow-up
Li et al.	2015	11	11 tibia	Pedicled	Massive allograft	Plate & screws	Radiological, bone scan 1 week post-operative
Jager et al.	2010	7	3 Tibia / 4 Femora	Free	Massive allograft	Plate & screws	skin paddle (5), radiological follow-up
Innocenti et al.	2009	21	21 Tibia	Free	Concentric tibia allograft	Plate and screws	Radiological, bone scan (17), skin paddle (4)
Houdek et al.	2016	18	9 Tibia / 9 Femora	Free	Cortical allograft	Bi-plating	skin paddle (6), radiological follow-up
Halim et al.	2015	10	7 Tibia / 3 Femora	Free	Massive cortical allograft	Plate & screws, external fixator in combination with screws	skin paddle + Doppler
Erol et al.	2015	7	1 Tibia / 6 Femora	Free	Full femur massive allograft	Plate & screws	radiological follow-up
Ceruso et al.	2008	31	31 Tibia	Free	Massive allograft	Plate & screws	radiological follow-up
Capanna et al.	2007	90	57 Tibia / 33 Femora	Free	Massive allograft	Plate & screws	skin paddle,, radiological follow-up

Complications

A wide variety of complications are described in literature after the reconstruction of long bone segments with the combination of a massive allograft and vascularized bone graft. A list of all complications is provided in Table 3. Significantly different complication rates were calculated ($p < 0.0001$) between the included articles. The complication rates ranged between 5.9 and 85.7 percent. Any complication major or minor has been taken into account. A total of 192 complications occurred in 96 patients in 15 of the included articles. Two studies were not included in the complication rate calculation because the numbers of patients with a complication were not available. We did not perform a pooled meta-analysis on complication rates, because the data on complication rates were divergent. Fractures were the largest group of complications, reported in 11 studies ($n=61$). These fractures included microfractures, stress fractures and re-fractures. Infections (superficial + deep) occurred in 10 studies ($n=23$). Nonunion was reported in nine studies ($n=30$) and delayed union in three studies ($n=5$) for which 15 additional surgical interventions were performed in order to achieve union. The use of pre or post-operative chemotherapy in oncological cases may contribute to the high non-union, infection and fracture rates [7, 13, 14, 16, 22, 23, 25]. Limb length discrepancy was reported in 37 cases, the reported length discrepancy ranged from 0.5 to 8 cm. Symptomatic Limb length discrepancies smaller than one centimeter were treated with a corrective shoe in 23 cases. Eight corrective osteotomies were performed for limb length discrepancies larger than one centimeter. Limb length discrepancy has been reported as a result of the ongoing growth of the contralateral side after the reconstruction. Two smaller but still frequently described complications were wound problems and Varus/Valgus deformity of the ankle at the fibula donor site. Wound problems occurred in six studies ($n=11$) and Varus/Valgus deformity's in seven studies ($n=17$). Rabitsch, Maurer-Ertl, Pirker-Fruhauf, Wibmeret al. [17] showed in their series a trend ($p=0.067$) of more wound healing problems in the tibial reconstructions compared to femoral reconstructions. On the other hand, the femoral reconstructions showed statistically significant more fractures than the tibial group ($p=0.0038$). See Figure 2 for complication rates in each study.

Table 3: Summary of all complications found in our study selection

Infection- superficial	DVT
Infection-deep	Arterial thrombosis
Deep sepsis	Flexion deformity big toe
Superficial necrosis	Varus deformity
Wound dehiscence	deformity of junction
Osteomyelitis	Valgus deformity
Hematoma	Limb length discrepancy
Pain	Clawed toes
Re-fracture	Hallux impairment
Microfracture	Joint instability
Stress fracture	Peroneal nerve palsy
Plate breakage	Allergic reaction
Fibula-allograft non-union	Recurrent tumor
Non-union	Amputation
Pedicle loss/avulsion	Metastasis

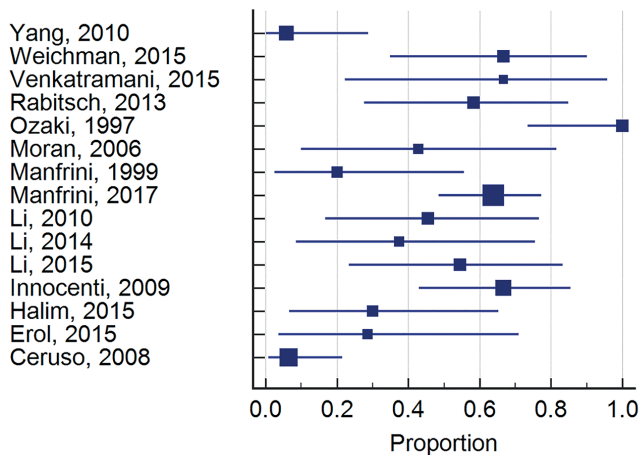


Figure 2: Complication rates by study. The calculated means are represented by the blue squares; each square differs in size because the size of the square represents the number of patients included in the study. The blue error bars represent the 95% CI.

Re-intervention

Re-interventions occurred because of fracture, non-union, infection, recurrence of the tumor, flap failure, deformity, and hardware removal. In 16 studies, re-intervention rates were known. Re-intervention rates ranged from 10 to 91.7 percent (Fig. 3). A statistically significant difference was found between each study ($p < 0.0001$). We included amputations because of an infection and or recurrence of tumor in our calculation of the re-intervention rate for each study.

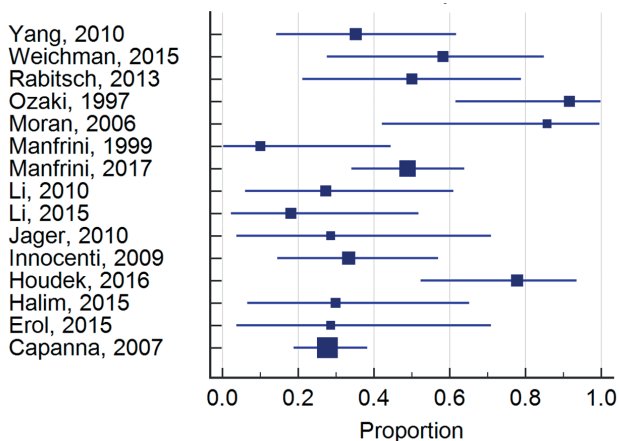


Figure 3: Re-intervention rates per study: calculated means are represented by the blue squares; each square differs in size since the size of the square is representation of the numbers of patients included in the study. The blue error bars represent the 95% confidence interval.

Union

Sixteen studies stated their primary union rates and described cases of nonunion or delayed union. Primary union rates between 66.7 and 100 percent were found. Based on these rates a meta-analysis was performed (Fig. 4). One publication [29] was not included in our meta-analyses since actual numbers were unknown. After pooling the data, a primary union rate of 86.5 (95% CI: 79.6 to 92.2) percent was calculated. Although the data could be pooled, there was a statistically significant difference in primary union rates between the studies ($p=0.003$). Manfrini, Bindiganavile, Say, Colangeliet al. [25] performed a case-control study comparing the outcomes of pedicled or free vascularized fibular grafts in combination with a massive allograft. This study showed no significant difference between the groups concerning complications or union.

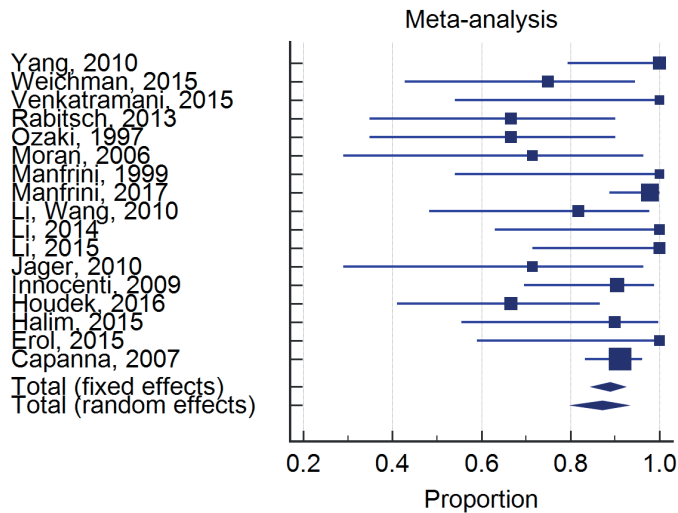


Figure 4: Meta-analyses on primary union rates: calculated means are represented by the blue squares; each square differs in size since the size of the square is representation of the numbers of patients included in the study. The blue error bares represent the 95% confidence interval. The blue Rhombus indicates the outcome of the quantitative statistical analyses for both fixed and random effect models. For this review we choose the random effect model (86.5, 95% CI: 79.6 to 92.2) since this is the right effect model if there is a lot of variation within the data.

Function

Eight studies used the Musculoskeletal Tumor Society Scoring System (MSTS-score) to measure their functional outcome after oncologic resection around the knee [13, 15, 16, 25-27, 30]. This scoring system ranges from either 0 to 30 points with 0 being a poor functional outcome and 30 point a good functional outcome. These studies showed an average MSTS score of 26.3 ranging from 24 to 29 (Fig. 5). Capanna et. al. [18] reported 72% excellent, 20% good, 5 % fair and 3% poor MSTS-scores. The remaining studies used other functional outcome rating systems such as the Mankin evaluation score [14, 22] and the Enneking system [19]. These data were not comparable.

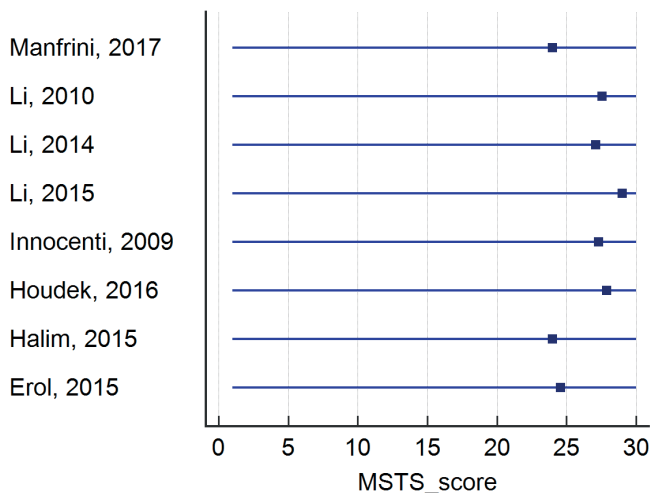


Figure 5: average MSTS score: calculated means are represented by the blue squares

Time to Full Weight Bearing

Nine studies described the average time to full weight bearing in 135 cases. The average time to full weight bearing in these studies was 11.28 months (95% CI: 7.99 to 14.6).

Amputation and recurrence

The oncologic studies reported 19 amputations in 331 reconstructions. These amputations were the result of local tumor recurrence or a severe deep infection or sepsis. A total of 21 reoccurring tumors have been reported and 23 cases of metastatic disease. Seventeen cases died from metastatic disease.

Discussion

Limb-sparing surgery has become important in the treatment of oncological and traumatic patients to improve their functional outcome [13, 14]. In long bone sarcoma patients 85-90 percent are candidates for limb-sparing surgery [1]. With the growing numbers of cases treated with limb-sparing surgery, a wide variety of reconstructive techniques for large bony defects has been described in literature. Massive structural allografts have been most frequently used for limb reconstruction but are associated with complications as allograft fracture, infection, and non-union due to the avascular nature of the graft [13-15, 32]. The Capanna technique was developed to add biological activity to the reconstruction to reduce these complications and induce revascularization of the allograft. The first reconstruction of an intercalary resection of the tibia with a combination of an external massive allograft a free vascularized contralateral fibular flap was done in 1989 [24].

Ozaki et al. [19] modified this technique in 1997 by using the ipsilateral fibula as a pedicled fibular graft. They modified the Capanna technique in order to reduce the risks and complications of a free flap, operation time and donor site morbidity. The Ozaki technique has inspired others to follow. Manfrini et al. [25] showed in a case-control study that there was no significant difference in outcome between a pedicled or free fibular flap combined with a massive allograft. Although there was a significant reduction in operation time in the pedicled group, as there was no need for microvascular anastomosis.

Capanna et al. suggested in 1993 that a minimal fixation method with small fragment screws combined with a long plate which did not span the entire defect was sufficient to achieve a stable reconstruction [21]. However, presentation of their long-term results in 2007, showed plate fixation to be more stable and achieve better union rates [18]. A rigid fixation with large plates and screws and even dual-plating have been thought to result in better union rates, lower fracture rates and less deformity related complications [13, 18, 19, 25].

Venkatramani et al. [7] published a case series of six cases reconstructed with the Capanna technique after post-traumatic defects in femurs. Their cases progressed into primary union (mean 6 months) without any additional surgical intervention to achieve union. Only one case of a deep infection occurred which was successfully treated with antibiotics. The authors claim that the reconstructions in traumatic cases have different challenges than those in oncologic cases. The problems associated with adjuvant chemotherapy and radiotherapy are absent, but the risk and incidence of infection are higher. Traumatic cases are often associated with open wounds, thus more susceptible to infection [7].

Vascularized bone autografts are well known for their biological activity, high hypertrophy and union potential [2, 13, 14]. Long-term radiographic studies of vascularized fibula autografts placed inside a massive allograft have shown progressive hypertrophy starting one month after reconstruction extending to 24 months [15, 27, 33]. Manfrini, Vanel, De Paolis, Malaguetiet al. [33] showed that the ongoing fibula hypertrophy inside of the allograft results in an endosteal reaction of the allograft. This indicates the ability of the allograft to adapt to stress due to revascularization of the allograft [33]. According to our results, fractures still frequently occur but successful fracture healing after ORIF or conservative treatment is the result of a process similar to normal fracture healing. This reflects the biological activity of the vascularized fibular autograft [13, 27, 33-35]. In addition, Innocenti, Abed, Beltrami, Delcroixet al. [27] showed the correlation between union of both allograft and fibula and the extent of fibular hypertrophy, reflecting the mechanical role of the fibula in the weight-bearing beside its biological role in union of the allograft.

The insertion of a vascularized fibula into a massive structural allograft possesses several downsides. For example, the structural allograft has to be adjusted to fit the vascularized fibula graft and allow hypertrophy which may reduce the strength of the allograft. Transfer of a vascularized portion of the fibular is associated donor site morbidity, such as motor weakness and sensory deficits in the foot, and ankle instability. Rare donor site complications reported are flexion contracture of the great toe, valgus deformity of the ankle, and stress fracture of the tibia. However, donor site morbidity is often acceptable in the majority of the patients due to preserved function in the reconstructed limb and low prevalence [21, 36].

CHAPTER 3

Free vascularized bone flaps necessitate microvascular repair of the nutrient vessels, which are associated with a low (<5%) risk of thrombosis of the micro anastomosis ^[37]. Failure to detect and revise early thrombosis of the anastomosis results in an avascular reconstruction. Non-vascularized reconstructions are submissive to creeping substitution. Thus, result is impaired strength and stress fractures over time ^[36]. Although the incidence of these complications is low, these results would be similar to reconstructions with a massive allograft alone.

This systematic review is limited by the level of evidence provided in the literature. All included articles had a level IV evidence except for one study with level III evidence ^[25]. Because of the difference in data reporting in each study, interpretation bias might have occurred in this review. To minimize this interpretation bias we used a standardized data extraction form. To compare the results of the included studies, subgroups were made for different outcomes. The main limitation of this study is the lack of comparative studies in literature.

The results of this systematic review show a wide variety of overall complication rates (5.9-85.7%). This is probably due to the limitation of taking the overall complication rate of each study, but it indicates the complexity of this type of reconstruction. Various factors contribute to the incidence of complications after reconstruction with a massive allograft combined with vascularized bone autograft. The most important factor we found is the use of perioperative chemotherapy or radiation ^[7, 13, 14, 16, 22, 23, 25] impairing the biological activity. According to the complication rates, re-intervention rates varied from 10-91.7 percent. Re-intervention often involved secondary bone grafting to achieve final union. Although this reconstruction method is associated with high complication and re-intervention rates our meta-analysis showed high primary union rates in 86.5% of the cases with good functional outcome (mean MSTs: 26.3) after this complex type of reconstruction.

The combination of a massive allograft with a vascularized fibula provides a single step reconstruction with good long-term outcomes in large segmental defects of various natures. This technique should especially be considered for treatment of large segmental defects in the lower extremity due to resection of primary bone tumors. Complications are manageable, albeit complication rates are high.

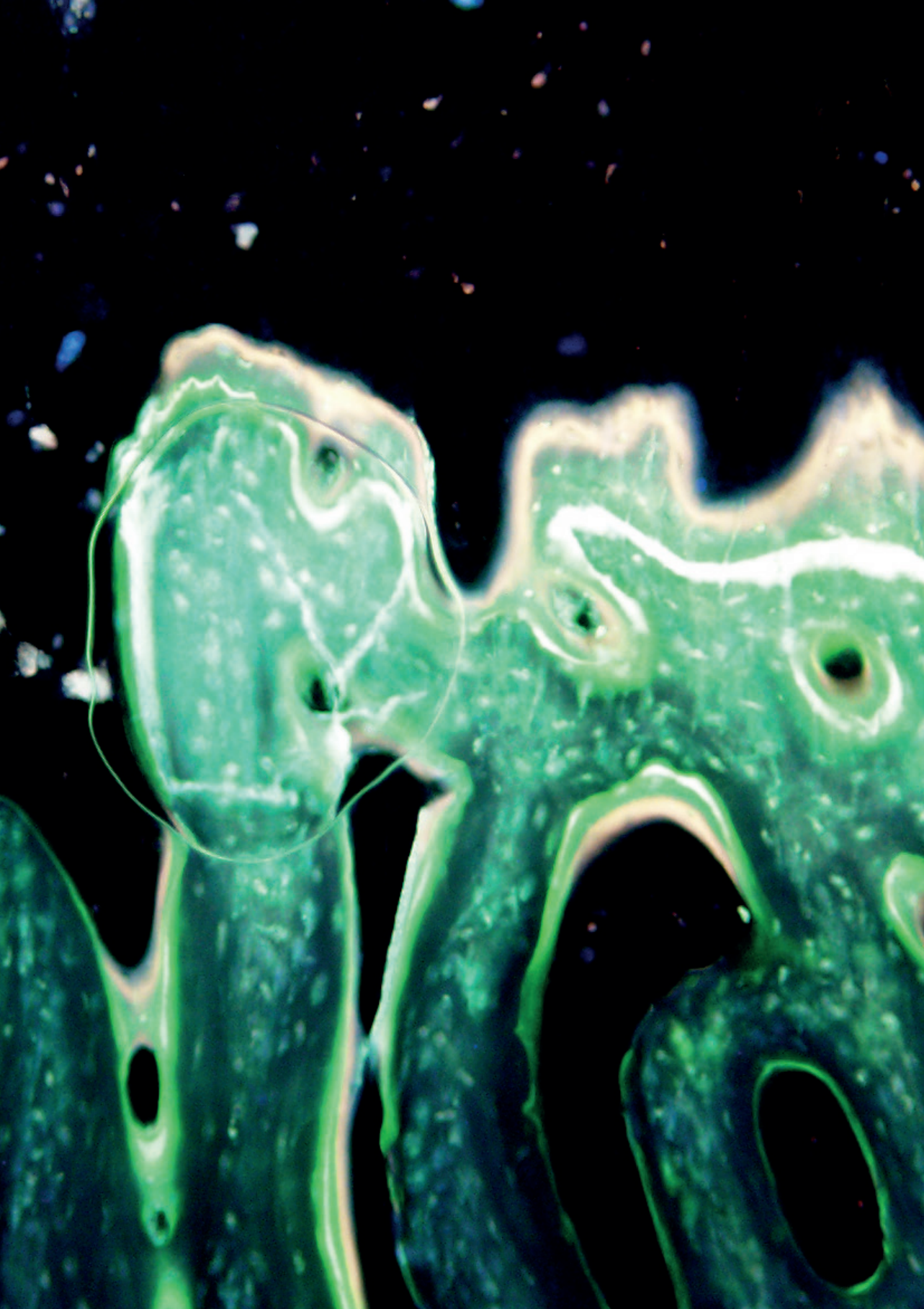
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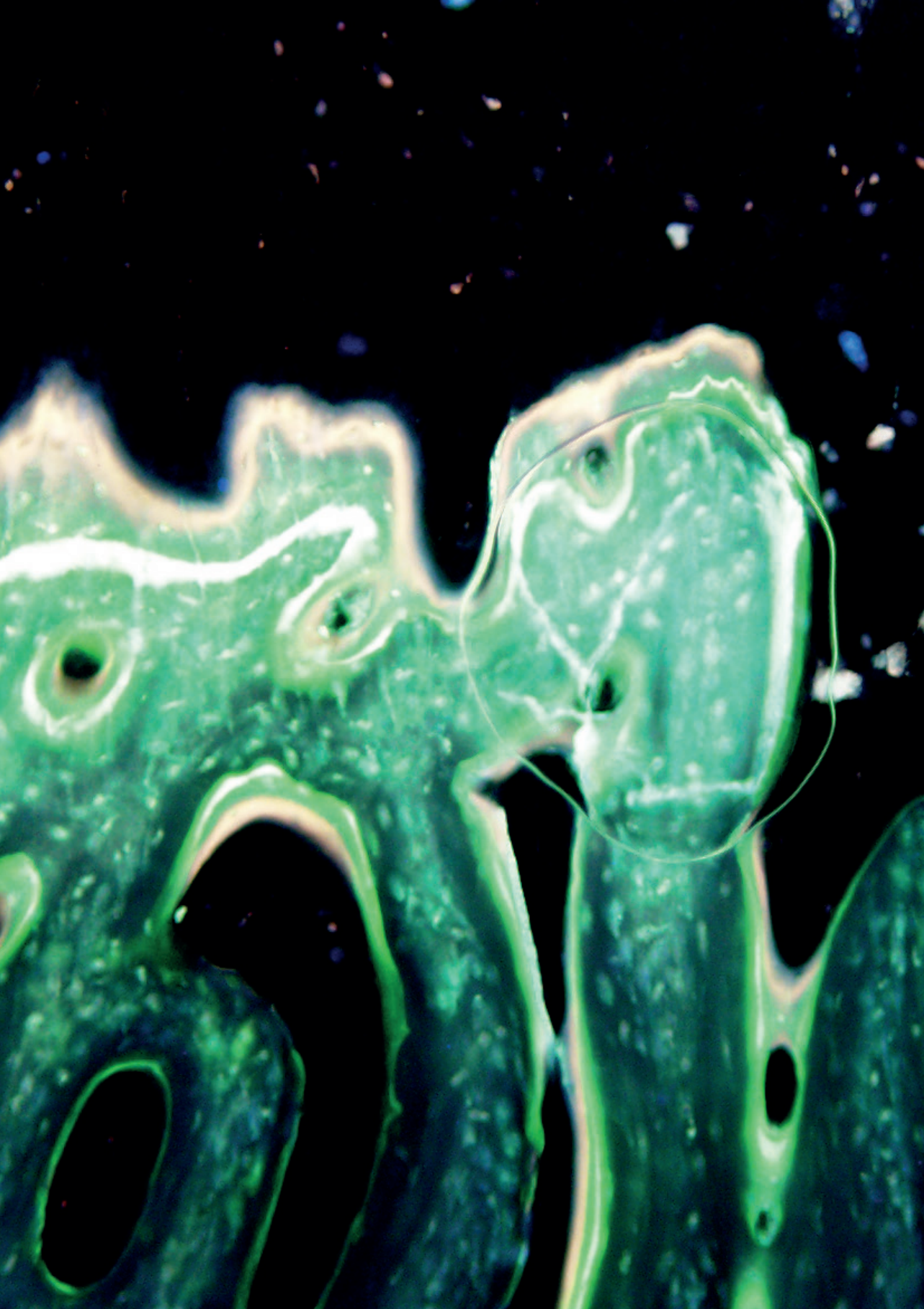
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PART 2





4

CHAPTER

**Outcomes of vascularized
bone allotransplantation with
surgical induced autogenous
angiogenesis in a large
animal model: bone healing,
remodeling, and material
properties**

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Abstract

Background: Bone vascularized composite allotransplantation (VCA) is a possible alternative for the treatment of large bone defects. Clinical application of VCAs is limited by the need for life-long immunosuppression (IS). We report an alternative method to maintain bone allotransplant viability in a large animal model without the need for life-long IS by using autogenous vessel-implantation.

Methods: Fourteen bone only VCA's were transplanted in a porcine tibia defect model with short-term IS. Two groups were used to evaluate the effect of the implantation of an autogenous arteriovenous (AV) bundle, therefore the only difference between the groups was the patency of the AV-bundle. We radiographically evaluated bone healing and allogenic pedicle patency. AV-bundle patency and union were evaluated with micro-CT. Bone remodeling was assessed with histomorphometry and material properties were evaluated with axial compression testing and cyclic reference point indentation.

Results: Two subjects did not reach the final time point. Twelve tibiae healed proximally, and 9 at the distal transplant-bone interface. Bone allotransplants showed their viability in the first 4-6 weeks by significant periosteal bridging arising from the transplant and maintained pedicle patency. Bone material properties were not affected by the implantation of an AV-bundle when compared to ligated AV-bundle controls but diminished compared to normal bone. Significantly higher bone formation rates resulted from the implantation of a patent AV-bundle.

Conclusion: New periosteal bone formation and subsequent bone healing results from blood flow through the microsurgically repaired nutrient blood supply, demonstrated by maintained allogenic pedicle patency. The implantation of a patent autogenous AV-bundle has no adverse effect on material properties, but a positive effect on bone remodeling of endosteal surfaces despite thrombosis of the allogenic pedicle. Bone material properties change after transplantation compared to normal bone, although 20-weeks survival time is relatively short for the final evaluation of bone material properties.

Introduction

Reconstruction of loss of large segments of bone is a challenging clinical problem. Current reconstructive options include the use of cryopreserved allografts, bone transport, prosthetic replacement, membrane-induced osteogenesis, re-implantation of autoclaved tumor bone and vascularized bone autografts, all with a significant incidence of complications and failure. Of these, the most successful are cryopreserved allografts and vascularized autogenous bone flaps. Banked bone may be matched to the bony defect, providing immediate stability without donor site morbidity. As they remain largely avascular over time, delayed or non-union is frequent, as are infection and late stress fracture^[1-7]. Vascularized autografts are limited primarily to the fibula and iliac crest for large skeletal defects. They better promote healing and resist infection, but often fail to provide sufficient strength and/or stability for function, with the risk of early fracture. Other methods are less commonly used, with higher rates of complications and/or significant morbidity. Transplantation of living allogenic bone has the potential to combine the same biological benefits as vascularized autografts with the mechanical advantages provided by size- and shape-matched cryopreserved banked bone segments^[8-10]. Practical use of vascularized composite allotransplantation (VCA) is limited by the need for life-long immunosuppression due to concerns of drug toxicity, expense, and complications with its use. The ability to maintain bone VCA viability without need for prolonged drug therapy would permit potential clinical use of size and shape-matched living bone for most any skeletal defect. The combination of microvascular repair of the allotransplant vascular supply with autogenous vessel implantation and two weeks of initial immunosuppression is a novel method to maintain allotransplant viability without the need for life-long immunosuppression. Successful results in small animal models have been encouraging^[11-13]. A large animal model with similar success could lead to clinical application for segmental bone loss^[11, 12, 14-22]. In this study, we report bone healing, remodeling and mechanical properties of bone-only VCAs using our previously described swine tibial defect model for this purpose^[17, 18].

Methods

Experimental Design

This study was approved by the Institutional Animal Care and Use Committee. All experiments were performed according to the established National Institutes of Health guidelines. Sinclair provided 21 Yucatan miniature swine (Sinclair Bioresources, LLC), matching 7 donors of two tibiae each to 14 recipient animals. All were matched by age (mean 5.8 months), size (15-35kg) and blood type (type A). All had swine leukocyte antigen (SLA) haplotyping prior to beginning the experiment, ensuring that no donor-recipient pairings were SLA-identical. This mismatch was sufficient to lead to rejection and VCA necrosis should the autogenous angiogenesis method fail. All 14 recipient animals had segmental tibial defects created and reconstructed with immediate VCA transplantation from the chosen recipient, described below. Animals were divided into two groups of 7 each, differing only in the patency of a second vascular supply. An autogenous cranial tibial arteriovenous bundle (AV-bundle) was passed into the medullary canal of the VCA segment. In Group 1 the AV-bundle was patent and in Group 2, ligated. The survival period was 20 weeks.

Short-term immunosuppression

All animals received a 2-week immunosuppressive triple therapy consisting of Tacrolimus (Sandoz Inc. Princeton, NJ), Mycophenolate Mofetil (Mylan Institutional Inc., Rockford, IL) and Methylprednisolone sodium succinate (Pfizer Inc., NY, NY). Tacrolimus and Mycophenolate were administered orally and Methylprednisolone intravenously. Immunosuppression levels were monitored by blood draws taken every other day from a central venous catheter. Dose adjustment was made to maintain a therapeutic level (Tacrolimus: between 5.0-15.0 ng/ml, Mycophenolate, between 1.0-3.5 mcg/ml). The methylprednisolone was tapered over the immunosuppression period.

Surgical Procedure [23]

Donor VCA harvest

The transplants were performed with two surgical teams, first harvesting two tibia segments from the left and right hindlimb of a single donor, then immediately transplanting the bone into matched tibial defects in one hindlimb of two recipient animals. In a series of prior hindlimb dissections, we determined that prolonging the vascular pedicle to include the femoral artery and vein greatly facilitated tibial VCA transplantation, permitting end-to-side arterial and end-to-end venous anastomoses of large vessels in the recipient hindlimb. This precluded using a single animal as both donor and recipient. Instead, in a non-survival surgery, one male Yucatan donor provided two tibial segments. This is a modification of our previously described method [23]. After initial induction of anesthesia with Tiletamine HCL + Zolazepam HCL 5 mg/kg IM (Telazol, Zoetis Inc, Kalamazoo, MI.), Xylazine 2 mg/kg IM (Xylamed, Bimeda-MTC, Cambridge ON, Canada), the animal was euthanized with Pentobarbital Sodium 0.22ml/kg (Vortech Dearborn MI). Using the previously-described method, a 3.5 cm tibial segment was harvested from each hindlimb. The segment harvested included the major nutrient pedicle of the tibia, located on the posterior-lateral border of the tibia immediately distal to the location of the tibial tubercle anteriorly and supplied by the caudal interosseous artery. An extensive dissection of its more proximal inflow to include to the femoral artery and vein provided large caliber vessels to facilitate its immediate transplantation.

VCA Transplantation

On the same operative day, two female Yucatan recipients were simultaneously anesthetized, then intubated and maintained with inhalation anesthesia (Isoflurane 1-3%). Prior to the incision, 1 gram of cefazolin (Hospira, Lake Forest, IL) was administered intravenously for infection prophylaxis. Tacrolimus and mycophenolate were administered by a gastric tube and methylprednisone, intravenously at this time. Exposure of the tibia and cranial tibial AV-bundle was as described previously [18], but modified with a second incision to expose the femoral vessels proximal to the knee. Exposure of the tibia and cranial tibial vessels was made through an antero-lateral incision. The cranial autogenous tibial AV-bundle was ligated distally and mobilized proximally from the surface of the interosseous membrane. With the use of our custom cutting jig, a 3.5cm tibial segment, identical in location to the donor VCA was removed. The donor segment was transplanted, and the vascular pedicle subcutaneously tunneled to the femoral artery and vein, where microsurgical

anastomoses were made (Fig. 1A). The AV-bundle was implanted in both groups but ligated in the control group (N=7). Rigid internal fixation was achieved with two orthogonal 2.7 mm 9-hole LCP plates (DePuy Synthes Vet, West Chester, PA) spanning the reconstruction (Fig.1B). A layered closure, use of Dermabond Prineo (Ethicon, LLC, San Lorenzo, Puerto Rico) and wrapping the hindlimb with Tegaderm and Ioban (3M Health Care, St Paul, MN) minimized the risk of deep wound infection. A central venous catheter (Hickman catheter, Bard Access Systems, Inc., Salt Lake City, UT) was placed in the internal jugular vein to enable intravenous drug administration and blood collection during the immunosuppressive period as described previously [18].

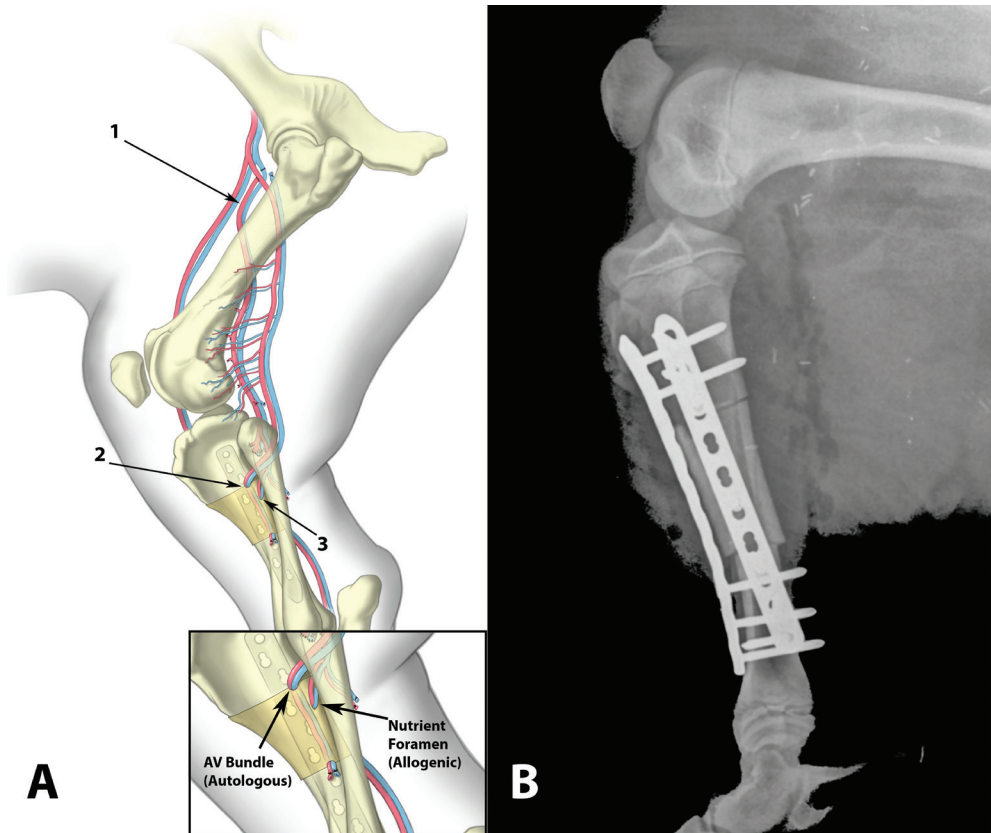


Figure 1: (A) schematic drawing of the orthotopic transplantation of a vascularized bone allograft model, (1) Allogenic pedicle with microanastomosis, (2) AV bundle entering the allotransplant into the intramedullary space, (3) Allogenic pedicle entering the nutrient foramen, (B) Post-operative radiographic image of the reconstruction.

Post-Operative Care

All animals received two weeks of prophylactic antibiotic therapy with Enrofloxacin 7.5mg/kg (Baytril, Bayer Healthcare LLC, Shawnee Mission, KS) and Ceftiofur 100mg/kg (Excede, Pfizer Inc, New York, NY). Direct weight-bearing was allowed after recovering from anesthesia. Daily observation permitted administration of appropriate analgesics, used until no evidence of pain was observed. The animals were individually housed and received standard feed and water ad libitum. Pigs were anesthetized at 2, 4, 6, 10 and 20 weeks for wound treatment, ultrasound assessment of vascular pedicle patency/thrombosis and radiography. At two weeks the immunosuppression was stopped, wound dressing changed, and the central venous catheter was surgically removed. Calcein (20mg/kg) and Oxytetracycline (20mg/kg) were administered by intramuscular injection 10 days apart prior to sacrifice.

Sacrifice Procedure

After the 20 week survival period all animals were anesthetized (Telazol + Xylazine, IM), and euthanized with intravenous administration of phenobarbital as recommended by the Panel on Euthanasia of the American Veterinary Medical Association and performed according to NIH guidelines under the direction of the Institutional Animal Care and Use Committee. The procedure was performed in a sterile manner. The femoral vessels were dissected and injected with Microfil (Microfil, MV-122, Flow Tech, Carver, MA). A Micro-CT of the whole tibia was made with all soft tissue attached, after removing the plates and screws. Next, the tibia was further dissected, and the allotransplant segment removed and divided into segments with an oscillating saw allowing multiple analyses of the allotransplant. For control purposes, the contralateral tibia was also removed, scanned, embedded and analyzed. Matched segments of contralateral normal and allotransplant bone were used for biomechanical analysis.

Imaging Studies

Ultrasound

Doppler ultrasound imaging (Vivid 7 dimension, GE Medical Systems, Horten, Norway) was used to evaluate the allogenic vascular pedicle patency over time. Since the allogenic vascular pedicle to the transplant was tunneled subcutaneously for anastomosis to the femoral vessels, biweekly assessment of the proximal pedicle patency was possible. We identified the proximal femoral artery and end to side anastomosis first with color Doppler imaging. By following the end of the anastomosis and lining up the skin mark, we were able to identify the subcutaneous vascular pedicle. The pedicle was considered patent if a triphasic Doppler signal could be generated and color Doppler imaging showed a pulsating subcutaneous artery. Studies were performed until pedicle rejection, determined at 2,4, 6 and 10 weeks postoperatively.

X-Ray

At 1, 2, 4, 6, 10 and 20 weeks after transplantation, lateral and anterior/posterior radiographs of the tibia were obtained. All images were digitally developed and scored using the radiographic evaluation of osseous healing and transplant incorporation system^[24]. This scoring system quantifies periosteal bridging, callus remodeling, union and transplant appearance at both of the host/transplant interfaces. Therefore, this system is reliable and suitable scoring system for bone healing after reconstruction with allografts or transplants^[19, 24]. Transplant appearance was scored as either no reaction of the transplant (0 points), resorption (-1 point) or periosteal reaction from the transplant (1 point). Scores ranged between 0-4 for each other evaluation point, with a maximum of 25 points. The scoring was done by an independent orthopedic surgeon at our institution.

Micro-Computed Tomography (micro-CT)

The experimental and contralateral tibias were both scanned using Micro-Computed Tomography (Inveon PET CT, Siemens Medical Solutions USA, Inc., Malvern, PA) at the termination of the experiment 20 weeks post-surgery. We used settings of 80 kV and 500uA, obtaining 180 projections with 188.5 μm thick sections at low magnification with a current version of the imaging software PMOD (PMOD Technologies, Zurich, Switzerland). The scans were used to quantify bone mineral density (BMD), visualize bony bridging and confirm AV-bundle patency. All measurement and imaging data calculations were performed using the current version of the measurement program AnalyzePro (Analyze, Mayo Clinic, Rochester, MN). Bone mineral density calculations were made after calibration with a hydroxyapatite phantom.

Biomechanical testing

Axial compression test

Bone quality was evaluated on a macro scale by axial compression testing to calculate the elastic modulus of the allotransplant and normal bone^[25]. The elastic modulus, defined as the ratio of stress to strain, is a representation of material stiffness. It serves as an indication of bone quality and its resistance to fracture. After harvesting the allotransplant was wrapped in a moist saline gauze and a 5mm transverse section obtained using a water-cooled Exakt saw (Exakt Technologies, Inc. Oklahoma City, OK). It was subsequently stored at -20°C for later testing^[17, 26]. Before testing, heights at three locations around the perimeter of each specimen were measured with a digital caliper (Absolute Digimatic, Mitutoyo, Kanagawa, Japan) and averaged to yield the specimen height. Specimen cross-sections were imaged on a digital scanner (CanoScan LIDE 100, Canon) at a resolution of 300 pixels/inch (PPI). Cortices were segmented by converting the scan to a binary image and cross-sectional area calculated using Image J (NIH, Bethesda, Maryland). Mechanical testing was conducted on a servo-hydraulic test frame (Model 312, MTS Systems, Eden Prairie, MN) instrumented with a 2500-kg capacity load cell. Specimen were loaded between two flat plates at a constant displacement rate of 1 mm/minute until reaching a maximum, sub-failure compressive load of 5000 N. The modulus of elasticity was determined from the slope of the linear region of the stress-strain curve.

Cyclic Reference Point Indentation

To evaluate bone properties on a micro scale, we performed cyclic reference point indentation (RPI). The Total Indentation Distance (TID) can directly assess the ability of the bone matrix to resist crack initiation and propagation. Normalized Indentation Distance Increase (NIDI) is inversely correlated with toughness and fracture ^[27]. After calibration, cyclic reference point indentation (RPI) testing was performed on a Biodent HFC system (ActiveLife Scientific, Inc., Santa Barbara, CA) using a cone-spherical test probe tip (BP2). Bone sections were irrigated with phosphate buffered saline (PBS) solution to maintain hydration. Twenty cycles of loading alternating between 0 N to 6 N at a frequency of 2 Hz were applied. Before testing each specimen, a polymethyl methacrylate block was indented to provide an internal reference standard. Since the tibia cross-section is roughly triangular, sites on each of the three sides of each specimen were selected for indentation. Each side was oriented such that the indenter made contact perpendicular to the surface. Axial measurements of the cortex were obtained by placing the specimen on a thin rubber sheet that prevented sliding during testing. One indentation at each site was performed. Results from testing were averaged for each cross-section to obtain a single value for each specimen.

Quantitative Histomorphometry

A 5 mm segment of the allotransplant was reserved for histological analyses, fixed in 10% buffered formalin for 48 hours, embedded in methyl methacrylate, sectioned using a diamond band saw, thereafter ground to 15µm-thick sections (Exakt Technologies Inc., Oklahoma City, OK). The unstained sections were analyzed with fluoroscopy (10X, Olympus BX51) since dual fluorescent labels were given prior to sacrifice 10 days apart. Both the endosteal and periosteal surfaces (six random fields on each surface) were analyzed to quantify label uptake: labeled surface (LS), single labeled surface (sLS) and double-labeled surface (dLS) per bone surface (BS), mineralizing bone surface (MS/BS). Based on these remodeling parameters we were able to calculate the bone formation ratio (BFR/BS ($\text{mcm}^3/\text{mcm}^2/\text{d}$)). Quantifications and calculations were done with the semi-automatic bone image analysis software (Osteomeasure; Osteometrics, Atlanta, GA).

Statistical Analysis

Since the collected data was collected from a low sample size ($N=14$) and not normally distributed, a non-parametrical test (Wilcoxon rank sum test) was used to detect a difference between the two groups (allotransplants with patent AV-bundle versus allotransplants with ligated AV-bundle). For the same reasons, a non-parametric (Wilcoxon signed-rank test) test was used to detect a difference between the operated and contra-lateral tibia. A Fisher's exact test was used to detect a difference in union at the time of sacrifice. All statistical tests were two-sided and differences were considered significant for p-values of <0.05 . Statistical analyses were performed using the statistical program JMP Pro 13.0.0 (SAS Institute Inc.). Statistical analysis was supported by the Center for Translational Sciences Activities (CTSA) at Mayo Clinic.

Results

Pigs were operated at a mean age of 5.8 months with a mean weight of 23.6kg. Over a 20-week period, pigs gained an average of 20.0kg. All animals were able to ambulate immediately with partial weight bearing, with observed full weight bearing without exception by four days (Fig. 2). During the 2-week immunosuppressive period, all pigs were in therapeutic range for Tacrolimus (mean 7.7ng/ml) and Mycophenolate (mean 1.3mcg/ml).

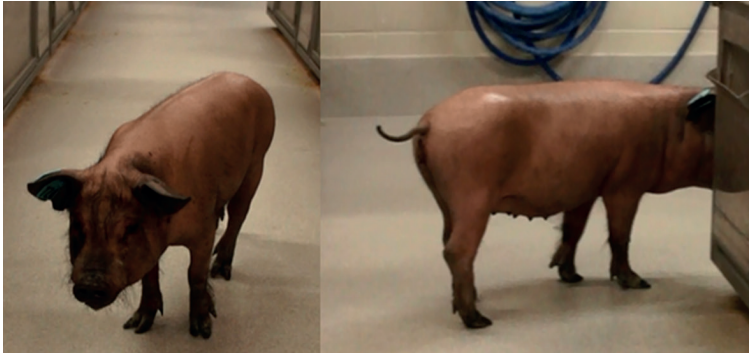


Figure 2: normal ambulation and walking 6 weeks after transplantation.

Vascular pedicle patency of the allotransplant

Doppler ultrasound examination of the transplant vascular pedicle at 2 weeks measured a mean peak systolic velocity of 26.87 cm/s and an end diastolic velocity of 8.68 cm/s (Fig. 3). All vascular pedicles showed a triphasic signal 4 weeks after transplantation. Nine of 14 vascular pedicles showed some non-triphasic blood flow at the 6-week time point, maintained in 3 animals at 6-weeks postoperatively. None of the allogenic vascular pedicles remained patent at week 10.

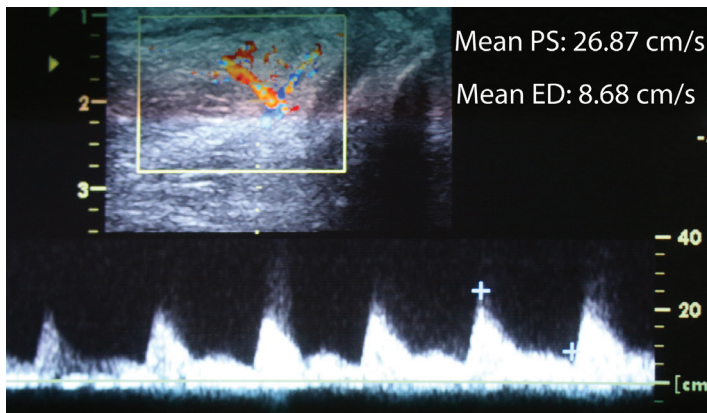


Figure 3: Doppler ultrasound imaging of the vascular pedicle at 4 weeks, showing pulsating blood flow with a mean peak systolic velocity (Mean PS) of 26.87 cm/s and a mean end diastolic velocity of 8.68 cm/s.

Arteriovenous (AV) bundle patency

Evaluation of the autogenous implanted AV-bundle was evaluated with Micro-CT angiography at the termination of the experiment (week 20). In the patent AV-bundle group (group 1) we found all of the implanted AV-bundles to be patent 20 weeks after transplantation.

Complications

Observed complications of the experiment were categorized as either minor or major. Major complications were those that required surgical intervention or resulted in termination of the experiment. One pig developed uncontrollable seizures (group 2), requiring euthanasia at 6 weeks. After full veterinarian autopsy, no focus for the seizures could be found. Another animal developed an abscess 6 weeks postoperatively at the medial malleolus (group 1). Although it resolved without apparent sequelae after debridement and antibiotic treatment, a deep infection with accompanying loss of bone alignment was confirmed at 20 weeks, caused by *Staphylococcus hyicus*. These two major complications required exclusion of the animals from further analyses.

Minor complications were defined those which resolved spontaneously or needed only medical treatment. In the first 2-4 weeks, six pigs developed a seroma in the inguinal area, which resolved spontaneously (3 in both groups). One pig developed a generalized skin rash (group 1), possibly due to an allergic reaction. We report a total of seven (7/12) minor complications and two (2/12) major complications in this study. We assessed twelve pigs for healing scores, biomechanical measurement, and bone properties, thus resulting in two groups of six pigs.

Bone healing

X-rays and CT-scans were evaluated for bony union on both the proximal and distal junction of the transplant using a scoring system devised for allograft healing [24]. All allotransplants showed new periosteal bone forming from both the allotransplant and native bones at both proximal and distal junction points 2 weeks following the tibial allotransplantation, serving to indicate transplant viability through the allogenic vascular pedicle (Fig. 4). Bone healing scores at the different evaluation time points did not show a statistically significant difference between the groups. A median score of 22.83 and 23.67 healing scores were found for group 1 and 2 respectively (Table 1). All proximal host-transplant interfaces (12/12) showed complete bone union 20-weeks after transplantation. Three distal host-transplant interfaces (3/12) showed incomplete distal union, two in group 1 and one in group 2. Serial radiographic evaluation showed the incorporation, remodeling and union of the allotransplant over time (Fig. 4). Insignificance in bone healing scores between the two groups indicates that the microsurgically repaired allogenic vascular pedicle, providing initial blood flow, was responsible for much of the observed bone healing and union.

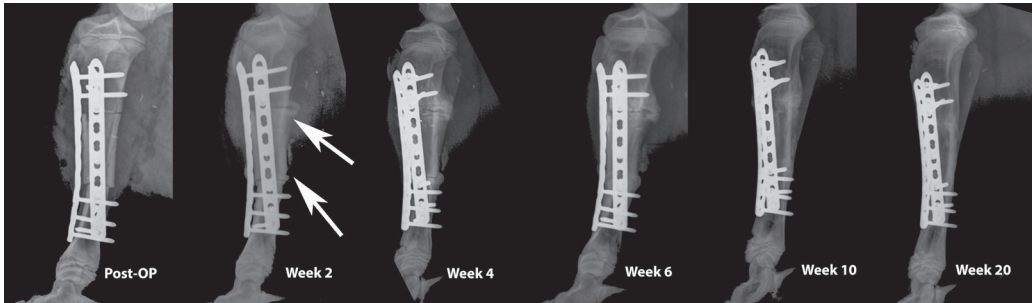


Figure 4: Lateral radiographs of the reconstructed tibia post-operative and at 2, 4, 6, 10 and 20 weeks after transplantation. The white arrows indicate the periosteal bridging arising from the viable allotransplant observed two weeks after transplantation.

Table 1: radiographic healing scores based on X-rays at 20 weeks*

X-RAY (scores)	Group 1	Group 2	P-value
Periosteal Bridging Proximal	4	4	1
Periosteal Bridging Distal	3.83	4	0.31
Callus Remodeling Proximal	4	4	1
Callus Remodeling Distal	3.33	3.66	0.60
Union Proximal	4	4	1
Union Distal	3	3.3	0.58
Transplant appearance	0.67	0.67	1
Total score (25 point = max)	22.83	23.67	0.60

*Values are given in means, 0-4 points could be given except for transplant appearance (0-1 point)

Bone Material Properties

All material properties of bone were evaluated 20-weeks after transplantation. Bone Mineral Density (BMD) measurements of the allotransplants with micro-CT showed the BMD was maintained in both groups. Bone porosity measurements showed a trend ($p=0.06$) towards a higher percentage of bone porosity in the allotransplants compared to the contralateral side (Table 2). The median elastic modulus (Mpa) of the allotransplants calculated by axial compression testing was lower than contralateral normal bone (Fig. 5), regardless of the patency of the implementation of an AV-bundle. The median elastic modulus significantly differed from contralateral normal control bone in both groups ($p=0.03$). No significant difference was found between the two intervention groups. Cyclic RPI of the allotransplant and of normal bone was conducted in the axial direction. The measurements showed a significant difference for TID and NIDI compared to contralateral normal bone (Table 3). No statistically significant difference could be found for the implantation of an AV-bundle. Cyclic RPI of the one 6-week survival allotransplant did not differ when compared to the 20-week samples. Assessment of bone material properties measured with micro-CT and biomechanical testing showed material properties of both allotransplant groups are diminished compared to normal (contra-lateral) bone at 20 weeks after transplantation.

CHAPTER 4

Table 2: Bone/Transplant properties on micro-CT evaluation

	Contra- lateral (cl) (N=12)	Group I (AV+) (N=6)	Group II (AV-) (N=6)	p-value cl-I	P-value cl-II	p-value I-II
Mean Bone BMD** (mg/cm ³)	478.46 (441.13-510.36)	431.65 (357.11-522.75)	371.97 (359.08-592.01)	0.16	1	0.87
Bone Porosity (%)	0.18 (0.13-0.27)	0.56 (0.43-0.75)	0.54 (0.30-0.98)	0.06	0.06	0.75

values are median and interquartile range ** BMD: Bone mineral Density; cl: contra-lateral normal bone

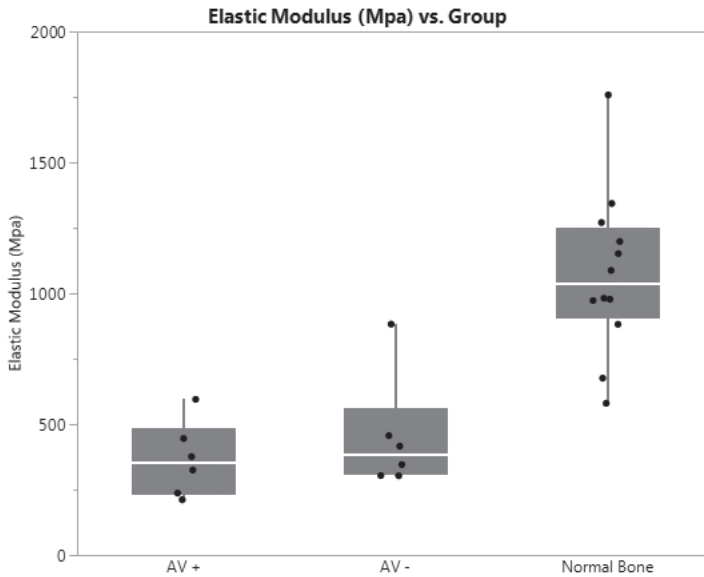


Figure 5: axial compression testing of allotransplants compared to contralateral normal bone expressed in elastic modulus (Mpa).

Table 3: Cyclic Reference Point Indentation after vascularized bone allotransplantation

Indentation	Contra-lateral (cl) (n=12)	Group I (AV+) (n=6)	Group II (AV-) (n=6)	p-value cl-I	p-value cl-II	p-value I-II
Total indentation dist. (TID)	79.33 (72.92-84.08)	103.5 (80.67-121.33)	132.92 (102.42-135.67)	0.03*	0.03*	0.63
Normalized Indentation Dist. increase (NIDI)	0.78 (0.70-0.80)	0.94 (0.89-1.53)	1.14 (0.87-2.15)	0.03*	0.06	0.63

values are median and interquartile range, * = significant

Quantitative histomorphometry

With the use of quantitative histomorphometry, we were able to quantify bone remodeling and bone formation. By quantifying the label uptake and the distance between the two labels, we were able to calculate bone formation and static and dynamic bone remodeling parameters (Table 4). Bone remodeling parameters measured on the endosteal surface with close proximity to the implanted AV-bundle showed an increase of the labeled surface (LS), single labeled surface (sLS), double-labeled surface (dLS), and mineralizing bone surface (MS/BS). Implantation of a recipient-derived AV-bundle significantly increased the bone formation ratio (BFR/BS) on endosteal surfaces in group1 compared to group 2 (Fig. 6). No significant differences in static and dynamic bone remodeling parameters were found on the periosteal surface of the allotransplants between groups.

Table 4: Quantitative histomorphometry analyses of the allotransplant in respect to the AV-bundle patency measured on the endosteal surface of the bone

	Contra-lateral (cl) (n=12)	Group I (AV+) (n=6)	Group II (AV-) (n=6)	p-value cl-I	p-value cl-II	p-value I-II
BFR/BS (mcm ³ / mcm ² /d)	340.57 (317.75-402.74)	512.55 (476.76-643.71)	379.29 (226.49-491.17)	0.06	0.69	0.03*

BFR= bone formation rate, BS= bone surface, values are median and interquartile range, *= Significant

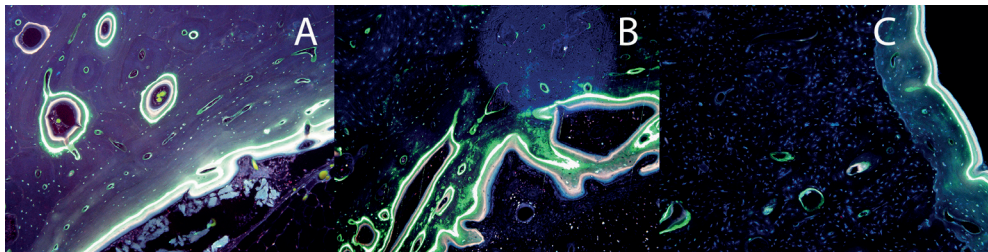


Figure 6: Representative images of fluorescent microscopy at 10X magnification on the endosteal surface of the allotransplants. Calcein and tetracycline label uptake in normal bone (A), Allotransplants with surgical induced neo-angiogenesis (B) compared to allotransplants without (C). A significant ($p=0.03$) increase in BFR/BS (mcm³/mcm²/d) was seen in the patent AV-bundle (B) group compared to the group with the ligated AV-bundle (C).

Discussion

A future alternative for skeletal reconstruction may be bone vascularized composite allotransplantation (VCA), as living bone allotransplants combine the advantages of cryopreserved allografts with the enhanced healing and remodeling potential of vascularized autogenous bone graft while minimizing the risk of a late stress fracture and resorption [8-10, 28, 29]. However, clinical literature and application of bone VCAs are limited since VCAs rely completely on lifelong immunosuppression with substantial side effects.

Experimental bone VCAs have been performed mainly in small animal models testing a variety of strategies to maintain viability without lifelong immunosuppression [11, 12, 20]. The Yucatan miniature swine tibial defect model has been proven to be this valuable and reproducible pre-clinical model to study the application of bone VCAs [23, 30]. Porcine models are also known for their comparable size, anatomy, physiology, and immunology to man [31-34]. The purpose of this study was to evaluate a larger animal VCA model and evaluate the clinical outcome, ambulation and quantify the bone characteristics after tibial VCA.

We used both axial compression and cyclic RPI evaluation of allotransplant material properties. Axial compression testing demonstrated allotransplanted bone to have a lower elastic modulus when compared to normal bone at 20 weeks. This is consistent with prior reports of bone VCA [30, 35]. Similar changes were seen with cyclic RPI calculations, consistent with the known changes in mechanical properties reported previously in response to transplantation or transfer [5, 9, 36, 37]. No adverse biomechanical effect of the implementation of an AV-bundle has been observed in both axial compression and cyclic RPI testing.

We found nine (9/12) of our tibia defect reconstructions healed at both the proximal and distal junctions 20 weeks after transplantation. Only three cases of incomplete distal union occurred. In clinical settings, secondary bone grafting would have been appropriate to achieve final union in these cases. Early unrestricted ambulation may have contributed to the distal non-union, although none appeared to have signs of painful weight bearing. For example, we have observed pigs “jumping” upon their pen door during feeding only four days after transplantation. We used rigid internal fixation with dual locked LCP-plates knowing that the pigs would immediately begin unrestricted weight bearing. Inability to limit activity in our pig hind-limb models is a potential explanation for the incomplete distal union. In clinical practice, no weight bearing would typically be allowed until radiographic healing has occurred.

We observed periosteal new bone arising from the vascularized allotransplant sufficient to form a bridging callous in both groups. This phenomenon is seen in vascularized autografts, but not in cryopreserved allogenic bone. The early development of periosteal callus in both groups is attributable to the patent microvascular anastomoses [24]. Despite the use of only 2 weeks’ immunosuppression, measurable blood flow was maintained for several weeks thereafter through the allogenic nutrient blood supply. The allogenic pedicle eventually lost its patency due to rejection and subsequent thrombosis, six weeks after transplantation. Rejection of the allogenic pedicle is the result of cessation of the immunosuppressive drugs followed by intimal

hypertrophy of the arterial wall [38]. The resulting vasculopathy causes transplant ischemia, as previously observed in clinical transplantation studies [38-40]. In this study, the neoangiogenic autologous vessels will not be similarly affected. This not only leads to drug-free VCA survival but results in gradual repopulation of the bone with autogenous osteocytes in areas of active new bone formation [15, 41-44].

We did not see any significant beneficial or adverse effects of AV-bundle patency on bone material properties. It may be that a 20-week evaluation point is insufficient to detect a significant effect on material properties [16, 45]. However, the implantation of a patent AV-bundle increased the bone formation rate measured 20 weeks after transplantation on endosteal surfaces. This indicates that the recipient-derived neoangiogenic circulation through the implanted AV-bundle does have a positive effect on bone remodeling. These findings are similar to our preliminary study [17]. The close proximity of the implanted AV-bundle to endosteal surfaces likely allows delivery of autogenous osteoprogenitor cells as well as osteogenic and angiogenic signaling molecules, improving bone remodeling and new bone formation. In a previous rat study of femoral allotransplantation using sex-mismatched donors and recipients, we demonstrated that the cells located in areas of new bone formation are autogenous rather than surviving allogenic cells [16].

Experimental results of orthotopic allotransplantation of bone in a large animal model have never been reported in these large numbers. The expense and technical difficulty of the allotransplantation procedure and subsequent evaluations are probable reasons. Bone healing with new periosteal bone formation from the allotransplant suggests short-term immunosuppression is sufficient to promote healing of large segmental defects reconstructed with living bone allotransplants. In addition, the implanted AV-bundle significantly improves the remodeling properties on endosteal surfaces of the allotransplant. Extensive remodeling of bone would be required to observe either a negative or a positive effect and may require a longer survival time to be adequately assessed [16, 45].

Ultimately, the goal of this research would be application of bone-only VCA to patient care. Clinical experience with bone and joint only VCAs is limited. The largest experience, from a German group, included three femoral diaphysis and five whole knee allotransplantations [46-52]. Another study transplanted a fibula from mother to child [53]. All but the latter report eventually failed due to infection, acute rejection, or chronic allotransplant vasculopathy [38, 47, 54]. None used autogenous angiogenesis nor current VCA drug regimens, however. The time may be ripe for carefully controlled experimental clinical trials of bone VCA reconstruction of segmental bone loss. Segmental defects in the extremities occur most commonly in limb-sparing tumor surgery, but also due to traumatic loss, infection or congenital anomaly. While long-term drug treatment would be contraindicated for some of these indications, short-term immunosuppression would likely allow bone VCA reconstruction using our method.

Conclusions

We demonstrate a technique useful for the experimental evaluation of bone only VCA with results consistent with an earlier preliminary report ¹⁷. Two weeks' immunosuppression is sufficient to maintain transplant viability with a triphasic Doppler signal in all animals until four weeks after transplantation. At six weeks the signal diminishes and after 10 weeks none of the vascular pedicles remained patent. Significant periosteal new bone formed by the allotransplant seen in both groups and the lack of significant differences in bone healing scores between groups suggests that this initial blood flow is responsible for much of the observed bone healing. Bone material properties of the allotransplant differ from normal bone but are not affected by the use of an AV-bundle. Thus, no adverse effect was seen by bone mechanical properties in the allotransplanted bone by the use of an AV-bundle. Bone mineral density was maintained after transplantation. Mean values were highest in the normal bone, followed by bone VCA treated with a patent AV-bundle. Both were larger than bone without a patent AV-bundle. Bone remodeling properties of the allotransplant were significantly better in the patent AV-bundle group. Bone VCA can safely be performed in a porcine animal model with reproducible results. More research should be performed to further evaluate the systemic and local immune response and repopulation of the allotransplant.

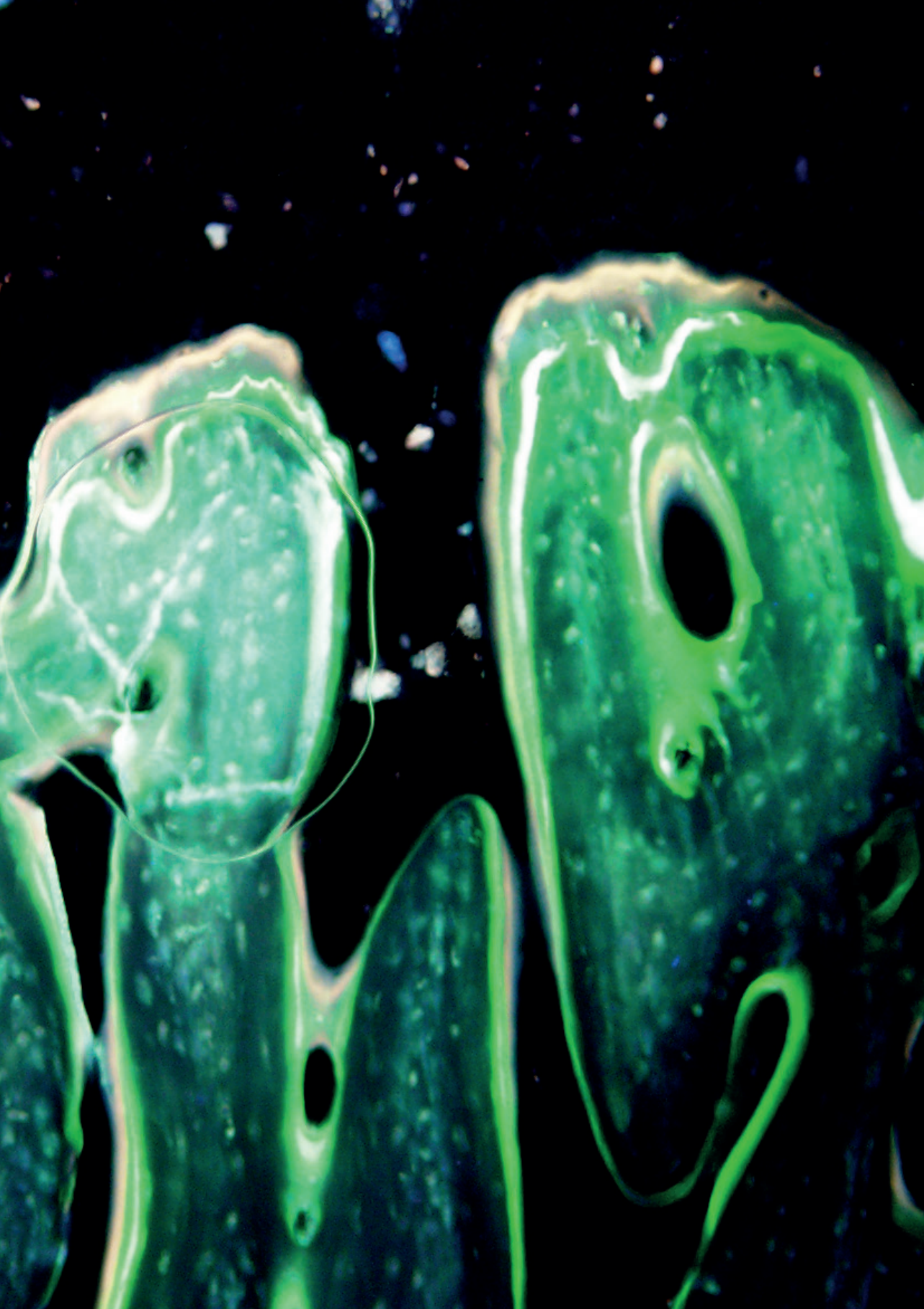
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CHAPTER 4

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5

CHAPTER

Neo-angiogenesis, transplant viability and molecular analyses of vascularized bone allograft transplantation surgery in a large animal model

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Abstract

Vascularized composite allotransplantation of bone is a possible alternative treatment for large osseous defects but requires life-long immunosuppression. Surgical induction of autogenous neoangiogenic circulation maintains transplant viability without this requirement, providing encouraging results in small animal models^[1-3]. A preliminary feasibility study in a swine tibia model demonstrated similar findings^[4,5]. This study in swine tibial allotransplantation tests its applicability in a pre-clinical large animal model. Previously, we have demonstrated bone VCA survival was not the result of induction of tolerance nor an incompetent immune system^[1]. Fourteen tibia vascularized bone allotransplants were microsurgically transplanted orthotopically to reconstruct size-matched tibial defects in Yucatan miniature swine. Two weeks of immunosuppression was used to maintain allotransplant pedicle patency during angiogenesis from a simultaneously implanted autogenous arteriovenous bundle. The implanted arteriovenous bundle was patent in group 1 and ligated in group 2 (a no-angiogenesis control). At twenty weeks, we quantified the neoangiogenesis and correlated it with transplant viability, bone remodeling and gene expression. All patent arteriovenous bundles maintained patency throughout the survival period. Micro-angiographic, osteocyte cell count and bone remodeling parameters were significantly higher than controls due to the formation of a neo-angiogenic autogenous circulation. Analysis of gene expression found maintained osteoblastic and osteoclastic activity as well as a significant increase in expression of endothelial growth factor-like 6 (*EGFL-6*) in the patent arteriovenous bundle group. Vascularized composite allotransplantation of swine tibia maintained viability and actively remodeled over 20 weeks when short-term immunosuppression is combined with simultaneous autogenous neoangiogenesis. These results are consistent with those seen in prior animal studies confirming maintenance of bone homeostasis and viability without the need of long-term immunosuppression.

Introduction

Current treatment options for large bone defects are associated with significant problems and complications. Vascularized bone autografts provide a good treatment option since they contain an intrinsic blood flow and remain viable over time. When compared to another common reconstructive method using cryopreserved allograft bone, their viability enables better healing, less risk of stress fracture, and a unique ability to remodel or even hypertrophy in response to loading. Vascularized bone autografts such as iliac crest and fibula are available for large defects although size and shape match with most segmental defects is poor. Stability and healing is improved when combined with a cryopreserved matched allograft segment^[6], but either method requires flap harvest and resultant donor site morbidity^[7-9].

Transplantation of living allogenic bone, a form of vascularized composite allotransplantation (VCA) would potentially combine the benefits of living bone and the ability to closely match the specific defect morphology without donor site complications. It has seldom been performed clinically, in part because allotransplant viability requires lifelong drug immunosuppression (IS). Its risks include organ toxicity, opportunistic infection and risk of neoplasm, ethically problematic to replace a non-life-critical structure.

Previous studies have demonstrated that bone VCAs may be maintained in small animal models without the need of long-term IS by switching the circulation of the transplant from allogenic to autogenous vessels, enabled by implantation of an arteriovenous bundle (AV-bundle) elevated from adjacent soft tissue and placed within the allotransplant. The allogenic nutrient vessels are repaired microsurgically at the same time, but only short-term immunosuppression is used^[1, 2, 4, 10-12]. The immunosuppressive period allows the nutrient pedicle to maintain transplant blood flow and cell viability. During this short period, angiogenesis from the AV-bundle develops to provide long-term bone perfusion. Because the anatomy and physiology of small animals differ from patients, we cannot necessarily extrapolate these data to humans. Our porcine tibial defect model more closely approximates clinical use, due to similarities with human physiology as well as transplanted bone size and shape^[4, 13]. This study reports transplant viability, formation of neoangiogenic circulation, bone remodeling and biologic activity after transplantation in a series of bone VCAs using this methodology avoiding the need of long-term IS.

Methods

Experimental Design

The Institutional Animal Care and Use Committee approved this study and all experiments were performed according to the established National Institutes of Health guidelines. Fourteen Yucatan miniature swine underwent orthotopic tibial bone VCA reconstruction in combination with surgical induced neo-angiogenesis and short-term immunosuppression. Seven living male Yucatan swine provided 14 vascularized tibia segments (VCAs). The allotransplant harvest, creation of the defect, transplantation, and fixation were performed largely as previously described, modified with proximal dissection of the vascular pedicle to include the superficial femoral artery and vein [13] (Fig. 1). One donor provided a pair of vascularized tibial segments for transplantation: one each from the left and right hindlimb. The ipsilateral hindlimb was used in each of two recipient swine. Donor and recipient were matched by age (mean 5.8 months), size (15-35kg) and blood type (type A). The animals were mismatched by pre-operative DNA sequence haplotyping to ensure five to ten class I and II swine leukocyte antigen (SLA) mismatches.

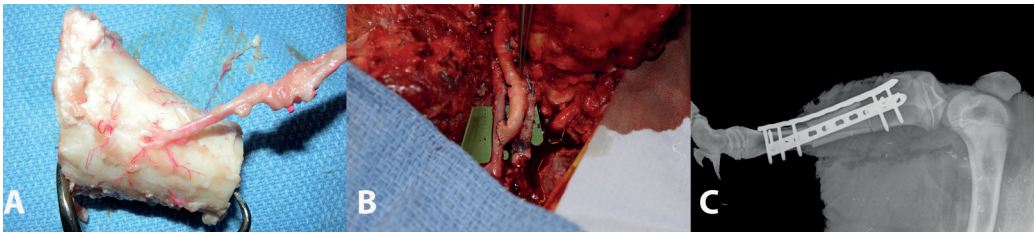


Figure 1: (A) 3.5 cm tibia segment with vascular pedicle showing the nutrient artery entering the graft by the nutrient foramen, (B) microsurgical anastomosis of the vascular pedicle to the femoral artery in a side-to-end fashion and the vein in an end-to-end fashion, (C) radiographic evaluation post-operative showing the reconstruction with internal fixation

Donor VCA Harvest

A single nutrient artery supplying the proximal tibial diaphysis is consistently found on its posterior surface 1-2 centimeters distal to the tibial tubercle. The nutrient pedicle is a branch of the caudal interosseous artery and vein. Briefly, the tibial VCA segment is harvested through an anterolateral incision, exposing the interosseous membrane and both cranial and caudal interosseous vessels. The proximal cut is made distal to the tubercle but proximal to the nutrient foramen. The distal cut is next made with a cutting jig to ensure uniform dimensions (Fig.1A). Proximal dissection of the pedicle to include the superficial femoral artery and vein completed the VCA harvest. The larger diameter of the femoral pedicle simplified the arterial anastomoses and enabled an end-to-end venous anastomosis not possible with the previously-described method (Fig.1B).

VCA Transplantation

The proximal tibia was exposed through an identical anterolateral hindleg incision. A segment of tibia was removed with the same cutting jig, from the same location as the donor VCA. A second incision in the medial thigh was used to expose the superficial femoral artery and vein, tunneling between the two incisions subcutaneously for pedicle passage. The VCA segment was placed into the defect and stabilized with dual locked compression plates (Fig. 1C), followed by end-to-side arterial and end-to-end venous anastomoses to the superficial femoral vessels. Surgical induced neo-angiogenesis was achieved by implanting an autogenous cranial tibia arteriovenous bundle (AV-bundle) into the medullary canal. Group 1 had a patent AV-bundle and in group 2 a ligated AV-bundle as a control. All animals were randomly divided into groups and received two weeks of immunosuppression postoperatively. This consisted of Tacrolimus 0.6-1.5 mg/kg (Sandoz Inc., Princeton, NJ, orally), Mycophenolate Mofetil 30-60 mg/kg (Mylan Institutional Inc., Rockford, IL, orally) and Methylprednisolone sodium succinate 500mg start-up dose (Pfizer Inc., NY, NY) intravenous for two weeks. Methylprednisolone was tapered over the immunosuppressive period. Immunosuppression levels were monitored by blood draws taken every other day from the central venous catheter. Dose adjustments were made to maintain a therapeutic level of Tacrolimus (between 5.0-15.0 ng/ml) and Mycophenolate (between 1.0-3.5mcg/ml). Only short-term immunosuppression was used, to test the ability of autogenous angiogenesis to maintain allotransplant viability long-term. All animals received prophylactic antibiotic therapy, appropriate analgesics and were monitored by staff veterinarians. The animals were individually housed, for a planned 20-week survival period. Unrestricted weight-bearing was allowed directly after the procedure.

Sacrifice procedure

A 20-week survival period was used in this study, chosen based upon our previous experience as sufficient to demonstrate substantial healing of the VCA segment as well as angiogenesis and resulting bone remodeling from a patent autogenous neoangiogenic blood supply^[4]. Practical considerations do not permit the many months or even years likely required for complete remodeling in a large animal model, nor multiple time points with large numbers of animals at each survival period. After the 20-week survival period all animals were anesthetized with Telazol + Xylazine IM, and euthanized with intravenous administration of Pentobarbital Sodium 0.22 ml/kg (Vortech, Dearborn, MI) as recommended by the Panel on Euthanasia of the American Veterinary Medical Association and performed according to NIH guidelines under the direction of the Institutional Animal Care and Use Committee. Both femoral arteries of the animal were dissected proximally, cannulated and flushed with heparin and saline. Later microangiographic analysis of neo-angiogenesis was enabled by injection with a contrast agent (Microfil, MV-122, Flow Tech, Carver, MA). After 45 min of curing, both the experimental and contralateral normal tibiae were harvested with sterile technique. A 5mm proximal segment was used for histology, and a more distal 2mm segment for PCR. Decalcification and micro CT angiography was performed on a 20mm mid-VCA section. The contralateral tibia was harvested, scanned and analyzed in the same manner for control purposes; we will refer to this as normal bone.

Histology

The selected bone segment was fixed in 10% buffered formalin for 48 hours, embedded in methyl methacrylate, sectioned using a diamond band saw and ground to 15µm thick sections (Exact technologies Inc., Oklahoma City, OK). The SRBS stained slides were used to analyze transplant viability by quantifying osteocytes, osteoblasts, and empty lacunae using light microscopy (20X, Olympus BX51) on the endosteal surface and periosteal surface (six random fields on each surface). Quantifications and calculations were done with the semi-automatic bone image analysis software (Osteomeasure; Osteometrics, Atlanta, GA). Bone viability was measured by calculating the percentage of either vacant or osteocyte-occupied lacunae using the Osteomeasure system. The percentage of empty lacunae was calculated by dividing the number of empty lacunae by the total (occupied and empty) lacunae X 100.

Micro CT angiography

The 20mm mid-VCA segment was fixed in 10% buffered formalin (48 hours) and decalcified over a 7 week period in Richard-Allan Scientific™ Decalcifying Solution (Thermofisher, Waltham, MA). Micro-CT scanning was performed using an Inveon PET CT scanner (Siemens Medical Solutions USA, Inc., Malvern, PA) using settings of 80 kV and 500uA and imaging software (PMOD Technologies, Zurich, Switzerland) at a medium-high magnification resolution. We used the BMA application of AnalyzePro software (Analyze, Mayo Clinics, Rochester, MN) to measure total transplant volume, cortex volume and medullary canal volumes. The segmentation portion of the application were used to separate the contrast-filled cortical and medullary vessels from surrounding bone. Capillary density within the allotransplants and normal tibiae was then calculated, reported as a percentage of total bone, medullary space and cortical bone volumes occupied by vessels.

RNA extraction, cDNA synthesis, and real-time quantitative PCR (RT-qPCR)

Another 2mm section of the allotransplant was cut with a cooled sterile oscillating bone saw. The section was cleared of excess soft tissue, snap frozen in liquid nitrogen and stored at -80 °C for later analysis. Bone sections were then individually pulverized in liquid nitrogen using the A11 basic analytical mill (IKA-Werke GmbH & Co. KG, Germany). RNA was extracted from the pulverized bone with PureLink RNA mini kit, TRIzol reagent and an on-column Pure link DNase treatment (Thermo Fisher Scientific, Cat no. 12813018A, 12034977, 12185-010, Carlsbad, CA). Quantification and determination of RNA purity were performed with a Nano-drop Spectrometer (ThermoScientific Nano-drop Technologies, Wilmington, DE) and absence of RNA degradation was confirmed by gel electrophoreses of the total RNA before conversion to cDNA. An iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA) was used for the reverse transcription reaction. Total RNA (200 ng) was mixed with nuclease-free water, iScript reverse transcriptase, and 5X reverse transcription reaction mix and converted to cDNA following the iScript protocol. RT-qPCR was performed to quantify the expression of target genes with iQ SYBR green Supermix using the CFX384 Real-Time detection system (Bio-Rad Hercules, CA). Transcript quantity measurements were normalized to *GAPDH*, and gene expression levels were

quantified using the $2^{-(\Delta\Delta CT)}$ method [14]. Primer sequences and genes of interest are given in table 1 (ThermoScientific, Invitrogen, Wilmington, DE).

Table 1: Primer sequence and name for each gene of interest

Gene	Full name	Sequence
Neo-angiogenesis		
<i>VEGF-A</i>	Vascular endothelial growth factor	5'-3': CTACCTCCACCATGCCAAGT 3'-5': ACACTCCAGACCTTCGTCGT
<i>EGFL-6</i>	Epidermal growth factor-like 6	5'-3': AGATGAACGGTGGGAAGATGG 3'-5': CAGATAAAGGGCCATCTGGA
<i>HIF-1A</i>	Hypoxia-inducible factor-1alpha	5'-3': TTACAGCAGCCAGATGATCG 3'-5': TGGTCAGCTGTGGTAATCCA
<i>CD-34</i>	Cluster differentiation-34	5'-3': GGAAACCACACCAGATGCTT 3'-5': AGGTCTGAGGCTGGACAGAA
Bone formation		
<i>CTSK</i>	Cathepsin K	5'-3': CGTGGCATTGACTCAGAAGA 3'-5': CCACAGAGACAGGTCCCACT
<i>RANKL</i>	Receptor Activator NF-kB Ligand	5'-3': TCACCAAACCAGCATCAA 3'-5': AAGTACGTGGCGTCTTGTC
<i>OPG</i>	The Tumor Necrosis Factor superfamily-11B (Osteoprotegerin)	5'-3': ATATCGGGCACATGAACCTC 3'-5': GGGGAAGTGGTACGTCTTGA
<i>BGLAP</i>	Bone Gamma-Carboxyglutamate Protein (Osteocalcin)	5'-3': TCACACTGCTTGCCCTACTG 3'-5': GGGTTGAGCTCACACACCTC
Housekeeper		
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	5'-3': ACACTCACTCTTCTACCTTTG 3'-5': CAAATTCATTGTCGTACCAG

Statistics

Since the data was collected from a low sample size (N=14), a non-parametrical test (Wilcoxon rank sum test) was used to detect a difference between the two groups (allotransplants with Patent AV-bundle versus allotransplants with ligated AV-bundle). For the same reasons, a non-parametric (Wilcoxon signed-rank test) test was used to detect a difference between the operated and contra-lateral tibia. All statistical tests were two-sided and differences were considered significant for p-values of <0.05. Statistical analyses were performed using the statistical program JMP Pro 13.0.0 (SAS Institute Inc.) and GraphPad Prism 5.03 for illustrations (GraphPad Software, La Jolla, CA). Power calculations were made by the division of biostatistics at Mayo Clinic using nQuery Advisor for outcomes of interest, including capillary density and osteocyte viability. This study was powered for an estimate of 80%, with significance level set at 0.05, to detect a minimal difference between the groups, based upon our previous studies of structural orthotopic bone allografts in rats and rabbits [Refs]. Due to complications during allotransplant harvest, only five animals in each group were included for gene analyses. Statistical analysis was supported by the Center for Translational Sciences Activities (CTSA) at Mayo Clinic.

Results

Surgical outcome

Our study used two experimental groups to evaluate the effect of the implantation of an autogenous AV-bundle on recipient-derived neo-angiogenesis, graft viability, and bone remodeling. The VCA studies were designed with a cohort of Yucatan swine (n=14) divided into two treatment groups (n=7 each; Group 1: tibial VCA + autogenous angiogenesis; Group 2: tibia VCA no autogenous angiogenesis control). All animals were able to ambulate with partial weight bearing immediately post-operatively, with full weight bearing by the fourth postoperative day in both groups. One abscess occurred at six weeks with a deep infection as a result. Six weeks after transplantation, another animal developed uncontrollable seizures. Extensive treatment was without success, requiring its sacrifice. Due to these complications, the two animals were excluded from this study for analyses. Thus, a final cohort of twelve pigs remained for analysis (6 in each group). No fractures were observed in these twelve animals and they all went into complete union of the proximal host-allotransplant interface, while only three pigs exhibited an incomplete union of the distal interface.

Neo-angiogenesis

To evaluate neo-angiogenesis within the allotransplant we performed a micro-angiography of the allotransplant to visualize the vascular pattern within the allograft. The micro-angiography showed that all AV-bundles (N=6) in group 1 were patent at 20 weeks. These implanted AV-bundles sprouted numerous neoangiogenic vessels within the medullary space and endosteal surface of the allotransplanted tibial segment. (Fig. 2A+B). The control group (group 2) had no significant medullary vasculature. In group 2, the source of bone blood supply came from the external cortical surface, consequent to contact the adjacent soft tissues (Fig. 2 C+D). The medullary vessel volume and calculated capillary density in the medullary proportion of the allotransplant were statistically significant higher in Group 1 than Group 2 ($p=0.04$) (Table 2). No significant difference could be found for the total and cortical proportions in vessel volume and capillary density.

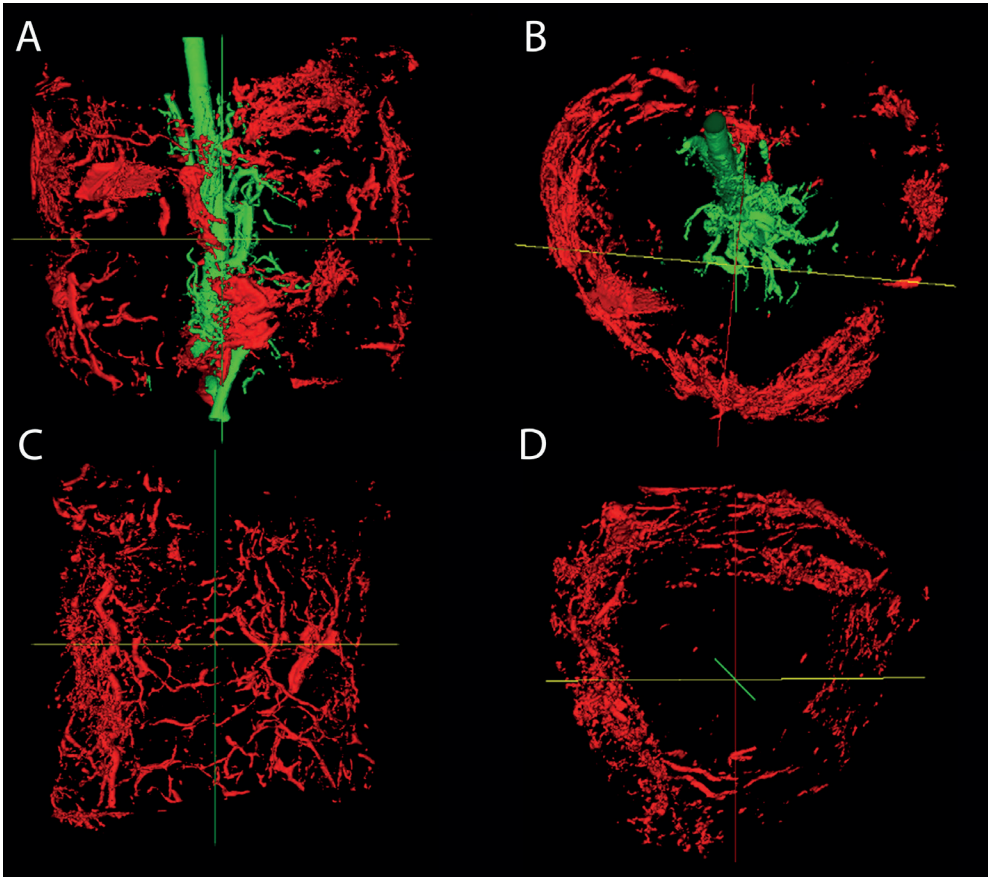


Figure 2: 3D reconstruction of the micro-angiography of the allotransplant showing the patent AV-bundle (green) in the medullary canal with neo-angiogenesis (A+B) in the longitudinal (A) and transverse view (B), in the ligated AV-bundle group showing only cortical vascularity (C+D) in red.

Table 2: Quantification of vessel volume in the allotransplant using micro-CT

	Group I (AV+) (n=6)	Group II (AV-) (n=6)	p-value I-II
Total Vessel Volume (%)	1.50 (0.45-2.18)	1.10 (0.87-1.72)	0.75
Medullary Vessel Volume (%)	1.18 (0.49-3.84)	0.19 (0.05-0.61)	0.04*
Cortical Vessel Volume (%)	1.45 (0.45-1.72)	1.31 (0.95-2.27)	0.63

Values are given in median and interquartile range, * significant

Transplant viability

We quantified osteocytes, osteoblasts, empty lacunae on the endosteal and periosteal surface of the transplant and calculated the percentage of empty lacunae as described in our methods. These parameters are important indicators for transplant viability. Calculations showed bone viability was better maintained with a patent AV-bundle, demonstrated by a significantly lower number of empty lacunae ($p=0.04$) on the endosteal surface of the bone (Table 3). No statistically significant differences were found between the two intervention groups in the number of osteocytes or osteoblasts. However, we did observe an increase in osteoblasts filling the endosteal surface of allotransplants in the patent AV-bundle group (Fig. 3B). No statistically significant differences were found on the periosteal surface of the bone.

Table 3: Histologic quantification of endosteal allotransplant viability

	Contralateral (n=12)	Group I (AV+) (n=6)	Group II (AV-) (n=6)	p-value cl-I	p-value cl-II	p-value I-II
N.Ob/B.pm (/mm ²)	10.68 (7.58-16.19)	19.67 (15.21-22.50)	13.62 (7.43-28.09)	0.005*	0.56	0.52
Occupied Lacunae (%)	83.98 (81.61-88.63)	88.24 (84.98-90.03)	78.35 (70-35-86.90)	0.15	0.22	0.04*

Osteocytes (N.Ot), Osteoblasts (N.Ob), Empty lacunae (N.Lac), Bone parameter (B.pm)

Values are given in median and interquartile range, * significant

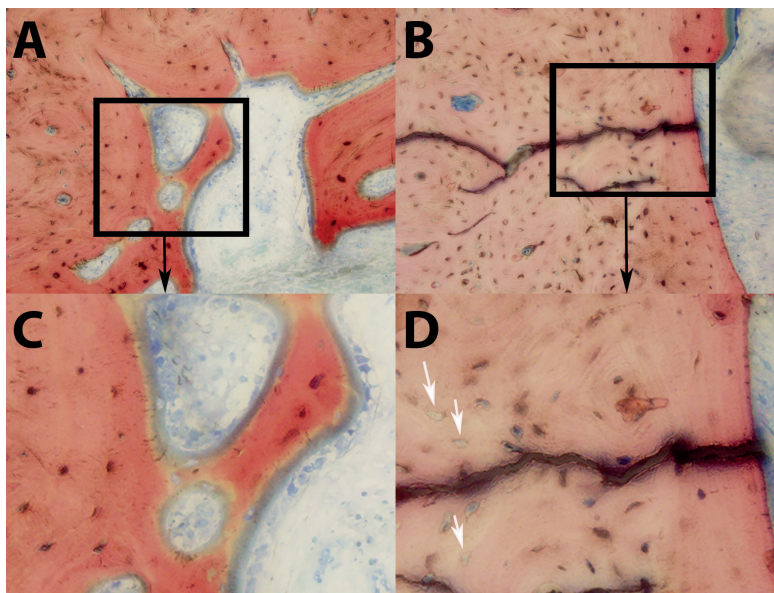


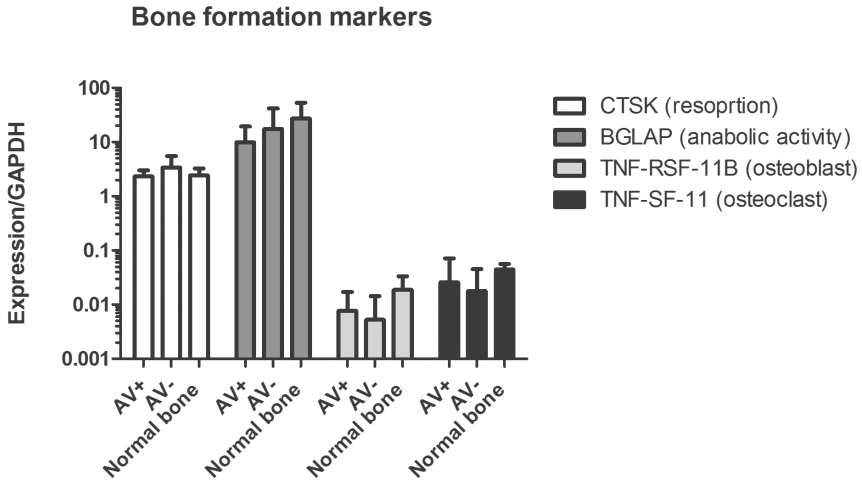
Figure 3: Representative histologic SRBS-images of the endosteal surface of the allografts with respect to the AV-bundle patency at 20X magnification. (A+C) allotransplant showing good osteocyte viability and osteoblast lining in the patent AV-bundle group, (B+D) Allotransplant with visible empty lacunae (white arrows) and a reduced amount of bone surface filled with osteoblasts in the control group (group 2).

Gene expression analyses

Bone homeostasis and remodeling markers

To examine the biological effect of vascularized allotransplantation on the bone homeostasis, we compared the expression of important osteoblast and osteoclast genes in normal bone and in our allotransplant groups. The Tumor Necrosis Factor superfamily of ligands (*TNFSF11*/ osteoclast marker) and receptors (*TNFRSF11B*/osteoblast marker) provide key paracrine communication signals for the differentiation of osteoclasts^[15]. These genes are also known as Receptor Activator of Nuclear factor Kappa-B Ligand and Osteoprotegerin (*RANKL/OPG*)^[16]. This interplay mirrors the important role of osteoblasts on osteoclast differentiation^[17]. Cathepsin K (*CTSK*), which encodes a lysosomal cysteine protease involved in bone remodeling and resorption, is predominantly expressed by osteoclasts. Bone Gamma-Carboxyglutamate Protein (*BGLAP*), also known as osteocalcin, is a protein secreted by osteoblasts that regulates bone remodeling and energy metabolism^[15]. Although we did find numerical differences between the groups, differences in important osteoclast (*CTSK*) and osteoblast (*BGLAP*) markers were not statistically significant. There were neither statistically significant nor biologically-relevant differences in biomarkers for bone homeostasis (*RANKL*, *OPG*) and bone remodeling (*CTSK*, *BGLAP*) between the groups or compared to normal bone. Thus, bone homeostasis and remodeling appear to be comparable between treatment groups as measured by gene expression analyses (Fig. 4).

A



B

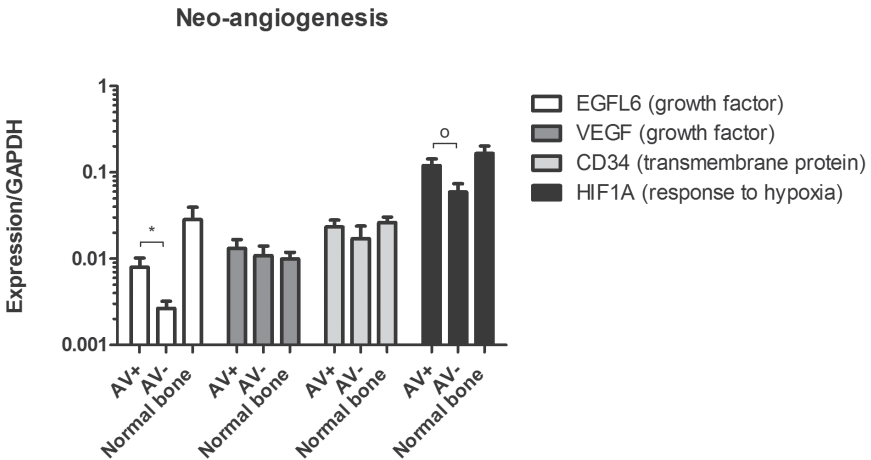


Figure 4: (A) Expression of bone formation markers, (B) expression of neo-angiogenesis markers with significantly * ($p=0.03$) higher expression of EFGL-6 in the patent AV-bundle group (AV+) and a trend O ($P=0.07$) towards higher HIF-1A expression in the AV+ group. Values are given in median and interquartile range.

Neo-angiogenesis markers

We examined the biological effect of different genes associated with neo-angiogenesis. Endothelial growth factor-like 6 (*EGFL6*), which is a member of the epidermal growth factor (EGF) repeat superfamily, promotes endothelial cell migration and angiogenesis by activation of extracellular signal-regulated kinase. In bone, *EGFL6* mediates cross-talk between endothelial cells and osteoblasts by this mechanism [15, 18]. Hypoxia-inducible factor 1 alpha (*HIF1A*) is a master regulator of cellular response to hypoxia in tissues. Vascular endothelial growth factor A (*VEGFA*) is one of the most important growth factors for regulation neo-angiogenesis. Cluster differentiation-34 (*CD34*) is a transmembrane protein on the vascular associated tissue and hence higher expression of *CD34* may correlate with increased presence of vascular tissue.

A biologically and statistically significant ($p=0.03$) increase in the expression of *EGFL6* was found for the patent AV-bundle group (Fig. 4). In addition, a trend towards higher expression of *HIF1A* was found for the implementation of an AV-bundle ($p=0.07$). *VEGFA* and *CD34* were expressed in all groups with no median significant difference between the groups. Thus, the implementation of an AV-bundle seems to have a positive biological effect on neo-angiogenesis markers (*EGFL6/HIF1A*) measured by gene expression analyses (Fig. 4).

5

Discussion

The ideal reconstructive method for large bone defects is one that functions immediately and is biologically equivalent to the missing bone segment. It should provide immediate strength, permit stable internal fixation, heal promptly and remain viable to resist infection, while resisting late structural failure by active bone remodeling. Transplantation of living allogenic bone is a potential candidate for such a 'perfect' solution—but only if the appreciable risks of long-term immunosuppression can be avoided. We have sought to replace the allogenic bone nutrient blood supply with an autogenous neoangiogenic blood supply to allow survival with only short-term immunosuppression. This has shown promise in prior small animal studies from our laboratory- but required a large animal study before consideration of this methodology in clinical practice. This porcine orthotopic tibial defect model was developed for this purpose. Our study which reports results at 20 weeks after transplantation surgery provides further insights into the potential future clinical utility of our method for bone vascularization.

In long bones, approximately 70% of the bone is vascularized by longitudinal endosteal blood supply and the remaining 30% is provided by periosteal blood supply [7, 19]. A vascularized bone autograft, such as the fibular free bone flap will maintain blood flow long-term, while a bone VCA pedicle will thrombose without sustained immunosuppression [1, 2, 4, 20]. This study advances previous work based on small experimental models by evaluating a novel method of VCA transplantation that requires neither systemic drug immunosuppression nor induction of donor-specific tolerance. Instead, surgically-induced autogenous neo-angiogenesis maintains VCA viability long-term [1, 2, 4, 5].

Previous published studies ^[2] ^[1] have provided results indicating that long-term graft viability can be maintained by surgical induced neo-angiogenesis and short-term immunosuppression. They transplanted vascularized allogenic femoral segments in a subcutaneous pocket in a rat model. Our current results on the effects of implanting autogenous AV-bundles corroborate these findings. The significant new finding of our current studies is that we are able to reconstruct a tibia defect in a weight-bearing pre-translational large animal model. Maintaining transplant viability in a large animal model has been previously described in a feasibility study ^[4]. The prior pre-clinical model with a 16-week survival period, quantified the same remodeling parameters as we did in our current model. In this study, we did observe three non-unions at the distal interface. The authors believe this could be due to the early mobilization and ambulation of the animals directly post-operative. In clinical setting these distal non-unions would be prevented by longer post-operative immobilization until healing has occurred. If a distal non-union would still occur, we could manage this complication with an additional cancellous bone graft.

The implantation of the AV-bundle within the bone in group 1 was hypothesized to develop a neoangiogenic autologous circulation in the allotransplant during the immunosuppressive period, as we have seen in rat and rabbit bone VCA allotransplants ^[1-3, 21, 22]. This is expected to maintain bone viability despite the expected thrombosis of the allogenic nutrient pedicle following stoppage of drug immunosuppression. We would further expect group 2 VCAs to have little or no endosteal cortical bone remodeling and less viable bone and marrow, again due to pedicle thrombosis but without the benefit of autogenous vascular angiogenesis. Evaluation of total and cortical bone vessel volume must include the substantial effect of well-vascularized tissue surrounding the VCA segment on the periosteal surface. The effect of the AV bundle in group 1, placed within the medullary canal, will be seen primarily in the medullary space and its primary effect expected to be on endosteal bone surfaces, at least in the first few weeks or months following transplantation. This is in fact what we found; the increased medullary vessel volume in group 1 had a significant positive effect on bone vitality on the endosteal surface of the allotransplant. The effect becomes less obvious when summed with the total bone and cortical bone volume.

Angiogenesis is required for bone development, growth, and repair. It is influenced by the local bone environment that involves cross-talk between endothelial cells and adjacent bone cells ^[18]. The role of *EGFL6* has been described in a calvarial osteoblastic cell culture (ex vivo) ^[18]. *EGFL-6* plays an important role in this cross-talk and promotes endothelial cell migration and angiogenesis. Although gene expression literature on angiogenesis markers after allotransplantation is scarce, we have shown a significantly higher expression of *EGFL-6* in our patent AV-bundle group. The higher *EGFL6* expression may have contributed to a higher medullary vessel volume. CD34 is a transmembrane protein in vascular associated tissue, therefore it is a parameter of the amount of vascular tissue when quantified by RT-qPCR. Quantification of total vessel volume showed a slightly higher volume of vessels (0.40%) in the patent AV-bundle group (Group1). Logically, one would expect the higher vessel volume would result in higher CD34 expression. Our results show a 1.36 higher CD34 expression in the patent AV-bundle group, but we could not appreciate a statistical correlation between CD34 and the higher vessel volume. *VEGFA* is considered an important neo-angiogenesis growth factor. We could not appreciate a positive effect of surgical angiogenesis on the expression of *VEGFA*. *HIF1A* should have a positive effect on *VEGFA* expression ^[23], although we could not appreciate this effect.

The discovery of the *RANKL/OPG* system and its role in the regulation of bone resorption has been relatively well described in literature since the 1990's [16, 17, 24]. In our results, the implantation of an AV-bundle does not seem to have a positive biological effect on bone resorption when we look at the *RANKL/OPG* interplay and the excretion of *CTSK* by osteoclasts when we compare them between the two intervention groups. We monitored putative bone anabolic activity using *BGLAP* gene expression as a biomarker. Overall, our molecular biomarkers for bone homeostasis were not statistically different upon implantation of AV-bundles. It is possible that the inability to detect differences in molecular bone parameters is due to technical issues, including biological considerations in the acquisition of RNA samples and technical complexities of RT-qPCR. For example, RNA was obtained by pulverizing a complete section of the transplant without separating medullary bone from cortical bone which may differ in bone turn over. Further research should analyze the bone homeostasis, bone remodeling and neo-angiogenesis by gene expression using endosteal and periosteal surfaces separately.

Our previous results in small animals have shown neo-angiogenesis from the AV-bundle reached the outer cortex in bone clearing studies [1, 2, 25]. Bone clearing protocols to visualize the contrast agent macroscopically have generally failed in larger animal models due to the thickness of the bone. Microangiographic quantification of the vasculature with micro-CT provides a good alternative, although it is a demanding and slow process. Microfil is useful, as it fills the microvasculature. Unfortunately, it has the same density as mineralized bone expressed in Hounsfield units. Micro-CT after decalcification provides reasonably good imaging of microvasculature in bone but is technically unable to follow the contrast agent from the AV-bundle through the cortical bone to its periosteal surface (Fig. 2). Ideally, we would have been able to follow the AV bundle through the transplant to the periosteal surface. This is a limitation of our study; future research should be performed with a higher magnification micro-CT. Alternatively, when we look at the evaluation of the microangiography and histology of the transplant, microfil reached into both endosteal and periosteal surface of the bone in group 1. It is probable that some vessels are from vascular ingrowth from the surrounding soft tissue.

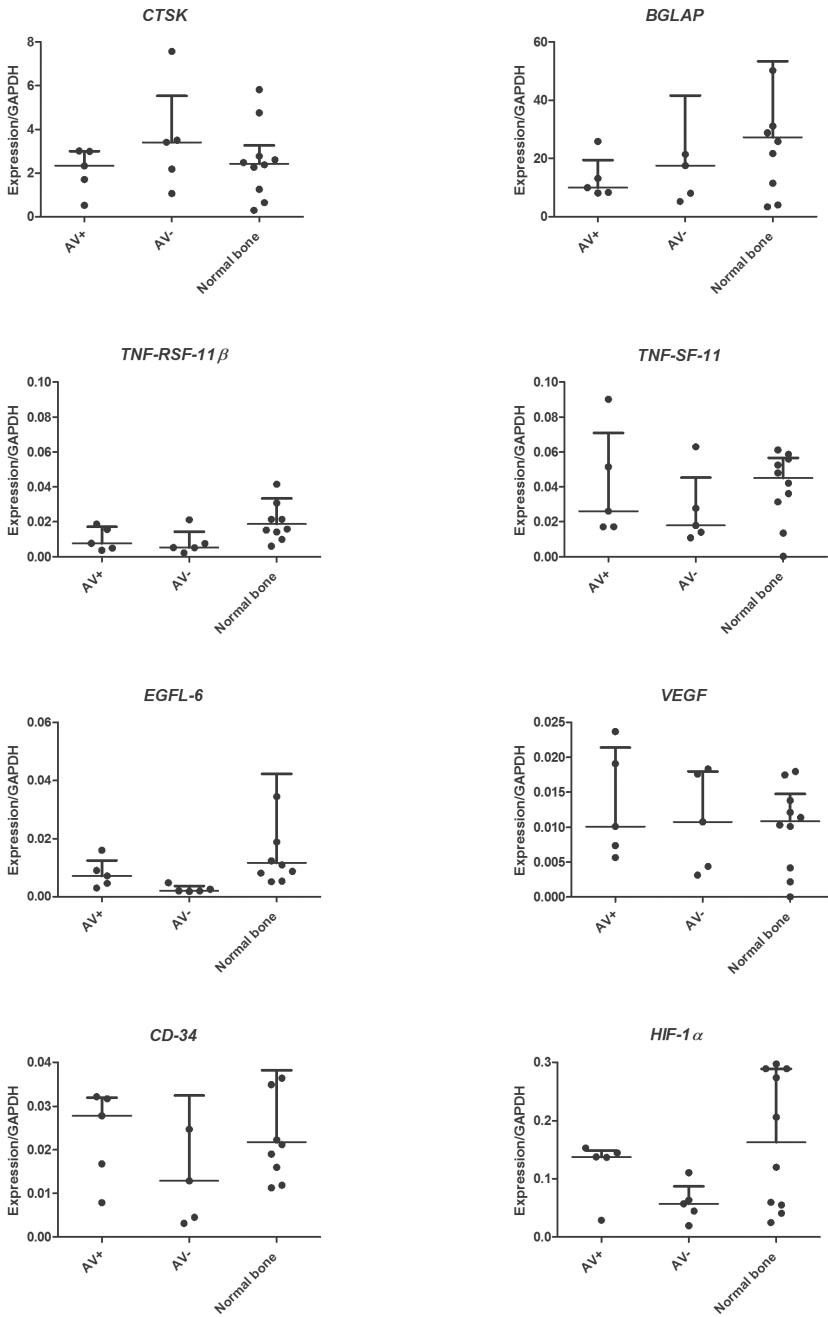


Figure 5: Supplemental PCR data, with individually plotted relative expression ratios for each animal. * = statistically significant ($p < 0.05$), O = trend towards difference between groups ($p = 0.07$)

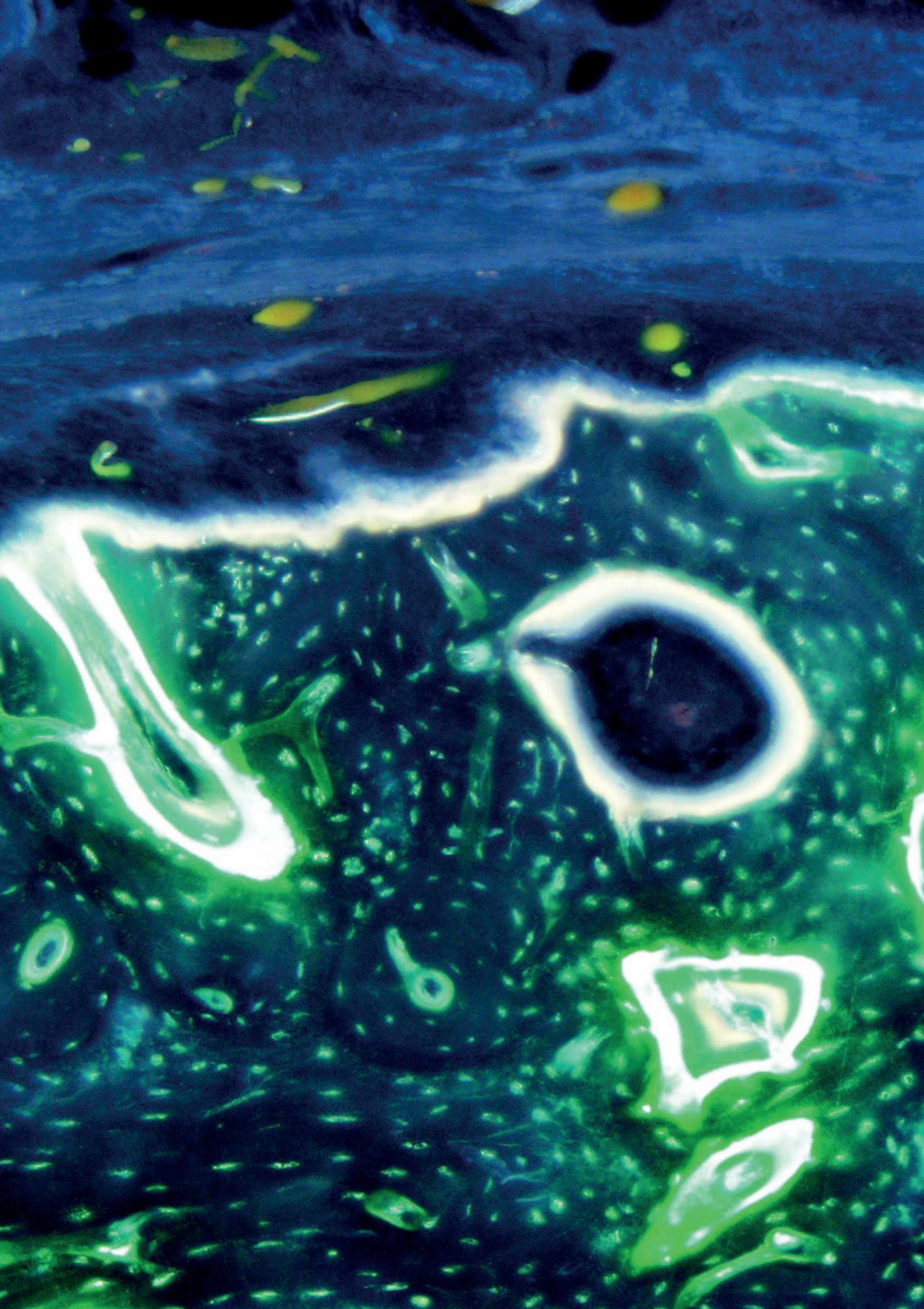
Conclusion

Living bone allotransplantation for the reconstruction of segmental bone loss in a preclinical model can be safely done with short-term immunosuppression due to recipient-derived neoangiogenesis with relatively low complication rates in a porcine model. The implantation of a patent AV-bundle generates an autogenous neoangiogenic circulation which maintains bone viability and maintenance of important osteoblast and osteoclast activity markers and a significant increase in the expression of endothelial growth factor like 6 (*EGFL-6*) after transplantation. However, further experimental research is needed to fully understand the exact behavior of VCA's with a larger sample size and longer follow-up. Additionally, the systemic and local immune response should be better understood before bone VCA may be safely performed clinically.

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CHAPTER

Transplant Chimerism in Porcine Structural Vascularized Bone Allotransplants

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Abstract

Background: Bone allotransplant viability can be maintained long-term by implanting arteriovenous (AV) bundles, creating an autogenous neoangiogenic circulation. Only short-term immunosuppression is required. This study investigates the origin of viable osteocytes observed in areas of active bone remodeling in orthotopically transplanted tibiae in a Yucatan mini-pig model.

Methods: Segmental tibial defects created in female Yucatan minipigs (N=14) were reconstructed with a matched vascularized composite allotransplant from a male donor. The circulation was microsurgically restored, with simultaneous autogenous AV-bundle implantation in group 1 (N=7). A ligated AV-bundle was implanted as a no-angiogenesis control in group 2 (N=7). After 20-weeks, repopulation of the allotransplant was assessed by real-time qPCR measurement of relative copy numbers of a Y chromosome-specific gene (*SRY*) and an autosomal housekeeping gene, ribosomal protein L4 (*RPL4*). A lower *SRY/RPL4* ratio demonstrates replacement of male allogeneic cells with female, autogenous cells in the sample. Genomic DNA was extracted from cross-sections of the allotransplant, liver and spleen. Additionally, areas of new bone formation within the allotransplant were sampled by laser capture microdissection. A comparison was made between groups as well as male control samples. RNA was extracted from bone as well, as a measure of metabolically active cells.

Results: Laser-captured areas of new bone formation in animals with both normal and ligated AV-bundles were found to have significantly lower relative copy numbers of *SRY* ($p=0.03$) than control specimens from male bone, indicating replacement by female (autogenous) bone-forming cells. Analysis of an entire segment of the allotransplant from Group 1 was similarly reduced ($p=0.04$), unlike that from Group 2. RNA expression of *SRY* was observed in both groups. No chimerism could be found in non-bone tissues (liver and spleen).

Conclusion: We observed a significant level of transplant chimerism in areas of new bone formation sampled by laser capture microdissection. The migration of autogenous cells including osteocytes was seen in both groups. Survival of some allogeneic (male) cells was also demonstrable. No microchimerism was found in liver and spleen.

Introduction

Segmental bone loss, often the result of limb-sparing resection of primary bone tumors, trauma, infection or failed primary reconstruction methods presents a challenging reconstructive problem. Current reconstructive options are problematic due to the risk of infection, non-union, stress fractures and implant failure. Living bone allotransplantation, a form of vascularized composite allotransplantation (VCA), is an alternative method with the potential to replace the missing bone with living bone closely matched in size and shape. Maintenance of VCA viability currently requires life-long drug immunosuppression. The significant expense of medication and monitoring, as well as risks of acute and chronic rejection, opportunistic infection, neoplasm, and organ toxicity, make this approach untenable for bone-only defects ^[1-3] ^[4, 5]. A method permitting drug-free bone allotransplant viability would quite possibly change clinical practice and improve patient outcomes.

In experimental small animal studies, we have previously shown that angiogenesis from arteriovenous bundles or fascial flaps within the medullary canal of bone VCAs will generate a neoangiogenic autogenous circulation. The allogeneic vascular pedicle reconstructed simultaneously, is required only during the initial postoperative period, maintained with 2 weeks of drug therapy. Despite subsequent pedicle thrombosis, the implanted autogenous vessels maintain bone blood flow and improve bone healing and remodeling. Transplant chimerism has been demonstrated in areas of post-transplant new bone formation in a rat femur model ^[6]. These levels increase with time, eventually resulting in a near-complete substitution of the allotransplant from allogeneic to autogenic ^[7]. In this study, we have applied the same methods to test whether microchimerism also occurs in bone-only VCAs used to reconstruct a major bone defect in a large animal model with long-term survival. Transplant chimerism and autogenous bone remodeling may lead to future clinical applications of vascularized bone allotransplantation.

Based on previous research, we hypothesized that bone-only VCAs will exhibit transplant chimerism, particularly in areas of new bone formation, facilitated by the development of an autogenous neoangiogenic circulation.

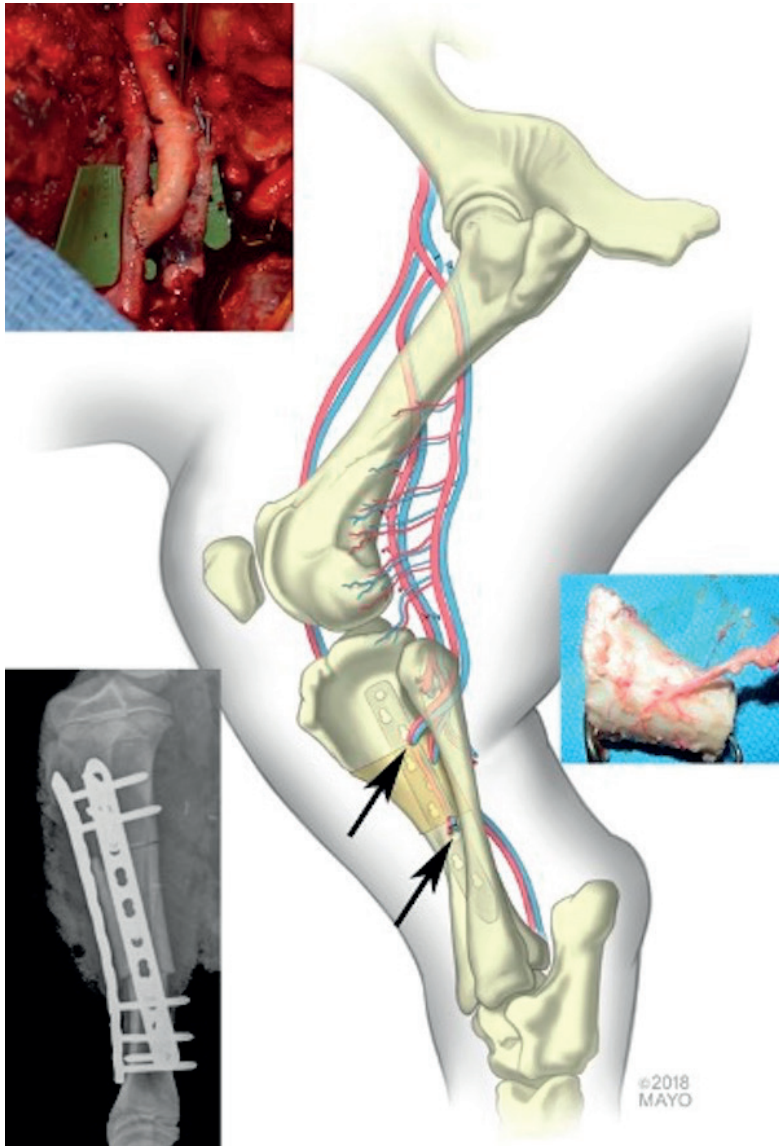
Methods

Transplantation Surgery

Vascularized bone allotransplantation was performed in a porcine tibial defect model. The Yucatan miniature swine with identical blood type and known swine leukocyte antigen haplotypes (SLA) were provided by Sinclair Bioresources, LLC. Male donors and female recipients were paired to result in an SLA mismatch and thus subsequent VCA rejection. A 3.5cm vascularized tibia segment was transplanted from the male donor into a matched hindlimb defect of a female recipient. Rigid internal fixation was made with dual locked plates. The nutrient blood supply was repaired with microsurgical anastomoses of both artery and vein. An autogenous cranial tibial arteriovenous (AV) bundle was simultaneously implanted within the medullary canal to create an autogenous neoangiogenic blood supply in group 1 (n=7) (Fig. 1). In group 2 (N=7), the arteriovenous bundle was ligated as a no-angiogenesis control. All animals received a 2-weeks' immunosuppression triple therapy regimen as previously described to maintain initial circulation through the microsurgically-repaired allogeneic nutrient blood supply during AV bundle angiogenesis^[8]. Subsequent VCA survival relied upon both the neoangiogenic endosteal circulation and ingrowth of vessels from periosteal surface contact, as the allogeneic vascular pedicle had thrombosed^[9]. The complete surgical procedure has been previously described^[10-12]. The experiment was terminated after a 20-week survival period. At fourteen and four days prior to sacrifice, Calcein (Sigma, St Louis, MO 20mg/kg, IM) and Oxytetracycline Hydrochloride (Vetrimycin, Boise, ID, 20mg/kg, IM) were respectively administered in order to detect new bone formation during the 10-day interval by fluorescence microscopy. Two pigs with surgical complications were excluded from the study. This study was approved by the Institutional Animal Care and Use Committee and performed according to established and National Institutes of Health (NIH) guidelines.

Sacrifice procedure

All animals were sacrificed after the survival period, as recommended by the Panel on Euthanasia of the Veterinary Medical Association and performed according to the NIH guidelines under the direction of the Institutional Animal Care and Use Committee. The allotransplant was harvested in a sterile fashion from the tibia and two 2mm cross-sections removed with a sterile cooled oscillating bone saw. Soft tissues were removed to obtain bone sections. Liver and spleen samples were acquired as non-bone tissues. All samples were snap-frozen in liquid nitrogen and stored at -80°C. Tissue sections were subsequently used for laser capture microdissection or nucleic acid extraction.



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Figure 1: illustration of the performed experimental reconstruction in a porcine hind-limb model. In the upper left-hand corner, the end-to-side arterial anastomosis is illustrated, in the lower left-hand corner a radiographic image is presented showing the rigid internal fixation. The black arrows point out the implanted arteriovenous bundle. On the right-hand side, the 3.5 cm allotransplant is demonstrated with the nutrient vascular pedicle.

Laser Capture Microdissection

Laser Capture Microdissection (LCM) was used to remove a small segment of newly formed bone from the area between the Calcein and Tetracycline double labels for subsequent PCR analysis. Bone cross-sections were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA Inc., Torrance, CA) at -20°C . Five μm sections were made on a Leica CM3050S cryotome (Leica Biosystems Inc, Buffalo Grove, IL), placed on a PEN-membrane slide (MembraneSlide 1.0, Carl Zeiss Microscopy GmbH, Gottingen, Germany) and stored at -80°C . For LCM, slides were dehydrated following the Carl Zeiss PALM-protocol for DNA handling. New bone formation was visualized by fluoroscopy at a 20X magnification. Areas of new bone formation were selected, and laser captured using the PALM MicroBeam system (PALM Microlaser Technologies AG, Bernried, Germany) with the PALM Robo 4.8 software (Carl Zeiss Microlmaging, Munich, Germany) (Fig. 2). Using a stable proteinase K as an extraction agent (Arcturus PicoPure DNA extraction kit, Arcturus Biosciences Inc.), microdissected tissue sections (total of $160,000\mu\text{m}^2$) were catapulted into the cap of a clean 0.6ml the tube containing 35mL of the extraction agent. Tubes containing the tissue and extraction agent were centrifuged and incubated following the manufacturer's protocol. The extracted genomic DNA was stored at -20°C prior to PCR analysis.

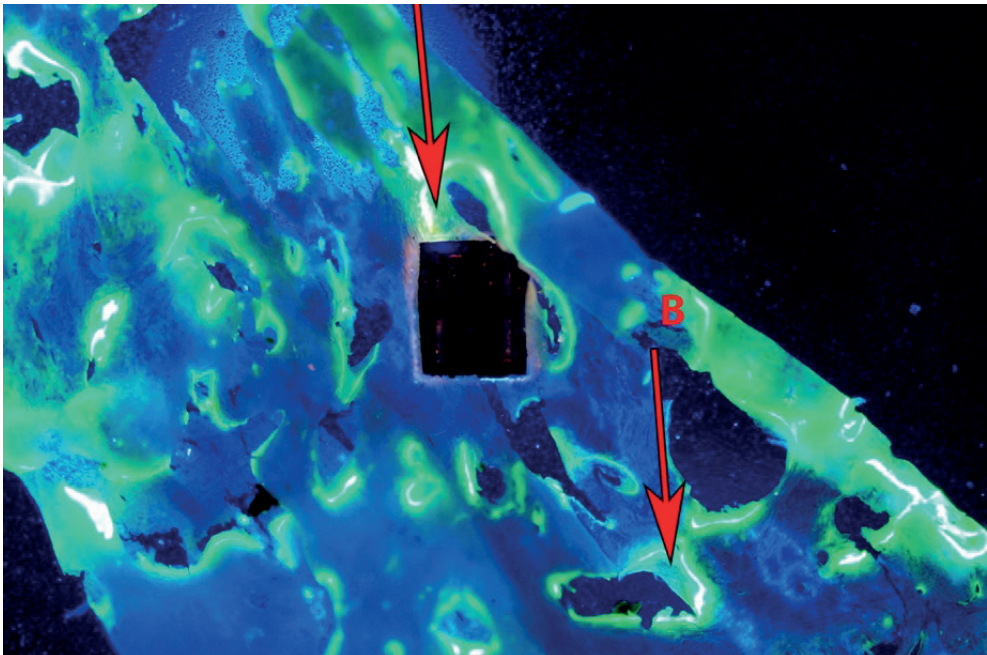


Figure 2: Fluorescence microscopy image at a 10X magnification of fresh frozen bone section after laser capture microdissection: (A) indicating the area dissected, which included newly formed bone. (B) indicating a similar area of new bone formation identified by fluorescence of Calcein and Tetracycline.

Whole tissue DNA/RNA extraction

Genomic DNA (gDNA) was extracted from bone, liver, and spleen of each animal. Frozen bone sections were pulverized in liquid nitrogen using a bone mill (A11 basic analytical mill, IKA-Werke GmbH & Co. KG, Germany). Liver and spleen samples were pulverized in liquid nitrogen using a mortar and pestle. Approximately 10mg of tissue was used for extraction, using a DNeasy Blood&Tissue kit (Qiagen, Cat. No. 69504, Hilden, Germany).

RNA was extracted from the pulverized bone with a PureLink RNA mini kit, TRIzol reagent and on-column Pure link DNase treatment (Thermo Fisher Scientific, Cat no. 12813018A, 12034977, 12185-010, Carlsbad, CA.). Quantification and determination of both gDNA and RNA purity were performed with a Nano-drop Spectrometer (ThermoScientific Nano-drop Technologies, Wilmington, DE). The absence of RNA degradation was confirmed by gel electrophoreses of the total RNA before conversion to copy DNA (cDNA). iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA) was used for the reverse transcription reaction. Total RNA (200ng) was mixed with nuclease-free water, iScript reverse transcriptase, and 5X reverse transcription reaction mix and converted to cDNA following iScript manufacturer's protocol.

We also extracted gDNA from female porcine bone and male porcine bone. These were used along with commercially available purified male and female porcine gDNA (BioChain Institute Inc., Newark, CA) for use as control samples. All gDNA and cDNA samples were stored at -20°C until PCR was performed.

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Quantitative Real-Time Polymerase Chain Reaction

Real-time qPCR was carried out to evaluate the extent of transplant chimerism as well as systemic microchimerism. Two separate analyses were performed in the VCA segment: newly formed bone obtained by LCM, as well as an entire cross-section of the transplant. We analyzed the ratio of male gDNA to an autosomal housekeeping gene (*RPL4*), expressed in both male and female cells. A similar analysis of extracted RNA demonstrated relative gene expression from viable male (allogeneic) and female (autogenous) cells.

Different primer sets were designed for gDNA analysis and RNA analysis (Table 1). The *SRY*-gene is located on the Y-chromosome and is therefore used to detect recipient or donor-specific cells in sex-mismatched models ^[13, 14]. Ribosomal protein L4 (*RPL4*) is a commonly used housekeeping gene in porcine models ^[15]. *SRY* primer sets (Integrated DNA Technologies, Coralville, IA) were designed for both the intronic and the exonic sequences of *SRY*. The intronic primer set was used to detect male cells (gDNA) and the exonic primer set (*SRY-e*) was used to detect RNAs produced by viable male cells ^[16].

CHAPTER 6

Real-time qPCR was performed using the iQ SYBR green Supermix (2X) and the CFX384 real-time detection system (Bio-Rad, Hercules, CA.) with 12 μ L reaction. Each PCR reaction consisted of 6.25 SYBR green, 0.25 μ L primers (20 μ m/ml), 3.0 μ L H₂O and 2.5 μ L sample. All samples were run in triplicate. Real-time PCR was followed by melt curve analyses with the following conditions: 15min denaturation at 95°C for one cycle, the 20s of denaturation at 95°C, 35s of annealing and extension at 72°C for 51 cycles followed by generation of a melting curve. Melt curves were performed from 60 °C to 95°C with an increment of 0.5°C. Real-time PCR products of samples and amplicons were run on a 2% agarose gel stained with ethidium bromide to confirm the proper size (Fig. 3). In addition, the PCR products were sent in for Sanger sequencing, and sequences lined up with the genes of interest.

Table 1: Primer sequences [16, 17]

Gene (ID)	Full name	Sequence
<i>SRY</i> (407740)	Sex-determining region Y protein	5'-3': AGTCAGTCACAGCCCAGTAA 3'-5': GGAAAATAAATGTGAGAAAG
<i>SRY-e</i> (407740)	Sex-determining region Y protein with exon	5'-3': TGGCGTAATTTGCGTCTTACT 3'-5': TCACCCCTTCTGAACCAGCTT
<i>RPL-4</i> (100038029)	Ribosomal protein L4	5'-3': AACGCTTTCATTGTGTGGTCTC 3'-5': CTCTGTGCCTCCTCGAAGAATG

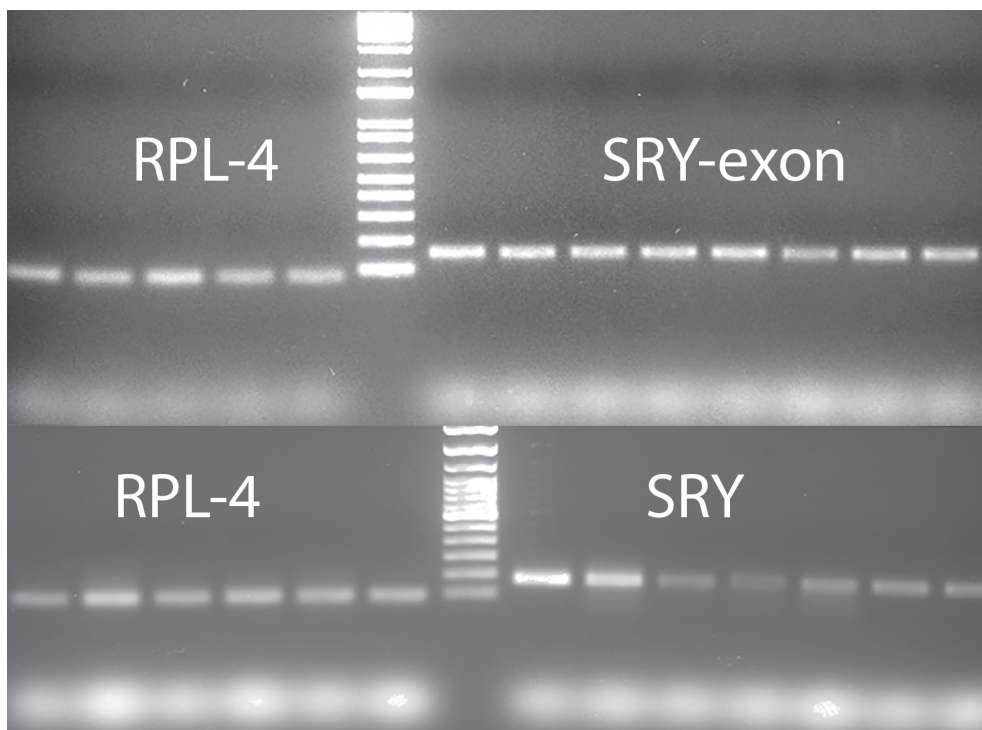


Figure 3: Electrophoresis 2% agarose gel confirming the right PCR product size for the different genes of interest.

Data analyses

The raw data from experimental samples produced by the CFX384 real-time detection system were transferred to Bio-Rad CFX Manager 3.0 software to calculate the efficiency curves. Transcript quantity measurements were normalized to *RPL4*, using the $2^{-(\Delta\Delta CT)}$ method [17] using Windows Excel. For DNA samples we refer to this ratio as the relative copy number of *SRY*. This ratio was measured for both male and female controls as well as the samples from both experimental groups. For RNA samples we calculated the relative expression ratio using the same method. In order to estimate the proportion of the allotransplant repopulated by female cells, we divided the relative copy number of *SRY* found in our samples by the mean relative copy number of *SRY* found in male control samples obtained from bone by the same method as the samples of interest and multiplied this by 100 ((Allotransplant copy number *SRY*) / (mean copy number *SRY* male bone)) x 100).

Standard Curve

A standard curve was run for *SRY*, *SRY-e*, and *RPL4* using the synthetic amplicon to evaluate PCR efficiencies. The calculation of the standard curve was done by using linear regression analyses. A 10-fold serial dilution was used for the dilution of the amplicon, resulting in dilution from 10^1 - 10^9 molecules/ μ L. Efficiencies ranged from 92.3% to 100%, coefficients 0.973-1.0 and standard curve slopes of -3.5.

Statistics

Since the data is collected from a low sample size, the Wilcoxon rank-sum test was used as a non-parametric test to detect a difference between the groups (allotransplants with Patent AV bundle versus allotransplants with ligated AV bundle and male control samples). All statistical tests were two-sided and differences were considered significant for p-values of <0.05. Statistical analyses were performed using the statistical program JMP Pro 13.0.0 (SAS Institute Inc.) and GraphPad Prism 5.03 for illustrations (Graph Pad Software, La Jolla, CA). Statistical analysis was supported by the Center for Translational Sciences Activities (CTSA) at Mayo Clinic.

Results

All animals were full weight-bearing after an average of 4 days. Twenty weeks after transplantation all animals achieved complete union of the proximal host-transplant interface. Only three incomplete unions were observed at the distal host-transplant interface. Two pigs with surgical complications were excluded from the study. One sample was excluded from analyses due to degradation of the DNA and RNA. Leaving 6 animals in groups 1, and 5 in group 2.

Quantitative real-time polymerase chain reaction analyses were carried out on male- and female-only gDNA control samples, to verify the specificity of the *SRY* primer sets and appropriate technique. The *SRY/RPL-4* copy number ratios calculated demonstrated the designed *SRY* primer sets to be specific to male cells. Additionally, H₂O-only samples were appropriately negative for DNA (Fig. 4). All extracted DNA and RNA samples expressed *RPL-4* in our PCR reaction.

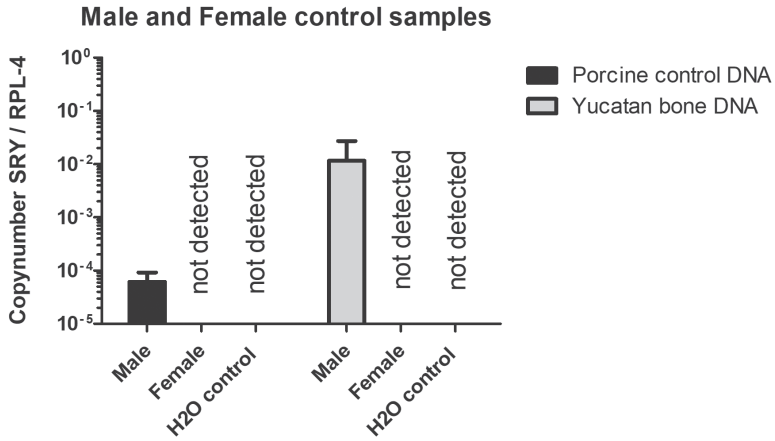


Figure 4: q-PCR analyses of gDNA from male and female control samples. The SRY -primer sets work on porcine DNA and Yucatan miniature swine DNA. SRY is not expressed in female control samples. RPL-4 was used as a reference gene.

New bone formation in allotransplants

We sampled areas of new bone formation between areas of double fluorochrome label using laser-capture microdissection. Significantly, only 3 of the 11 (group 1) allotransplants expressed any *SRY*. New bone was formed in the remainder entirely by autogenous (female) cells. We also found significantly lower relative copy numbers of *SRY* in both groups, when compared to male bone controls ($p=0.004$) (Fig. 5). The mean relative copy number *SRY* in the patent AV bundle group (Group 1) was 1.42×10^{-4} (SD 2.3×10^{-4}), and 1.29×10^{-3} (SD 2.89×10^{-3}) in group 2. Male control levels were much higher: 6.0×10^{-2} (SD 1.0×10^{-2}).

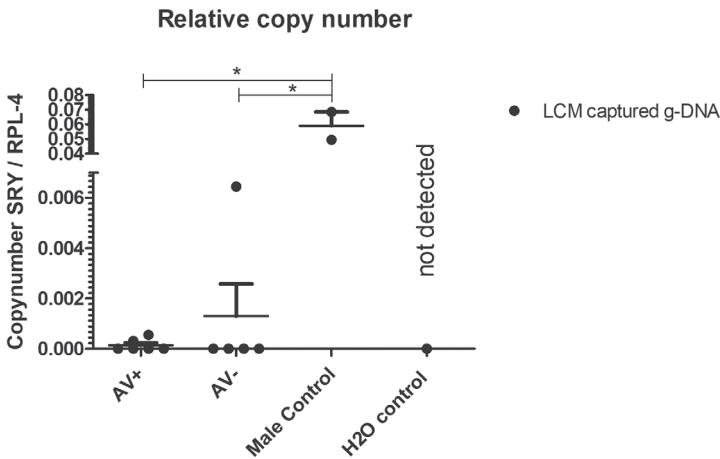


Figure 5: q-PCR analyses on gDNA from areas of new bone formation captured by LCM. Only three LCM samples *SRY* was detected, resulting in a significantly lower ($p=0.04$) copy number compared to male control samples in both intervention groups. Mean relative copy number *SRY* in the AV+ group: 1.42×10^{-4} (SD 2.3×10^{-4}), AV- group: 1.29×10^{-3} (SD 2.89×10^{-3}), Male control: 6.0×10^{-2} (SD 1.0×10^{-2}). No *SRY* or RPL-4 was detected in H2O control samples.

Cross-section of allotransplant

Genomic DNA recovered from pulverized cross-sections was also studied. *SRY* gene copy numbers were similarly expressed as a ratio to the housekeeping gene. Ten of 11 DNA samples obtained from a cross-section of the allotransplant expressed *SRY*. The mean relative *SRY* gene copy number was 1.97×10^{-7} (SD 2.91×10^{-7}) in group 1, 2.32×10^{-6} (SD 2.99×10^{-6}) in group 2, and 1.0×10^{-2} (SD 1.0×10^{-2}) in male controls. Group one had a significantly lower *SRY* copy number than male controls (Fig. 6). Group 2 copy numbers did not reach a similar significance when compared with either male control bone or Group 1 data (Fig. 6).

Calculation of the percentage of *SRY* present in the allotransplant compared to male control samples, showed no significant difference in the presence of the *SRY* gene between the two groups, although the patent AV bundle group had a lower mean percentage (mean 1.7×10^{-3} , SD: 2.5×10^{-3}) compared to the ligated group (mean: 1.7×10^{-2} , SD: 2.4×10^{-2}). The lower proportion of *SRY* found in allotransplant cross-sections reflects a smaller percentage of male cells in the representative section of the allotransplant and thus the extent of repopulation (Fig. 7).

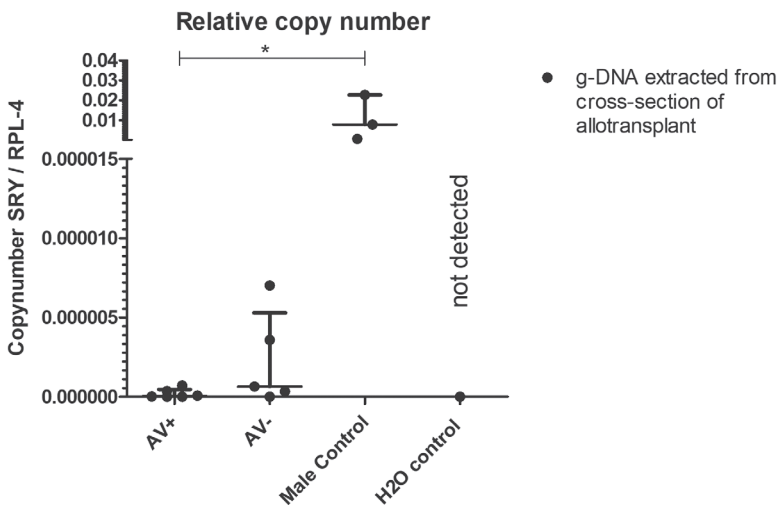


Figure 6: q-PCR analyses of gDNA extracted from pulverized transverse bone sections. *SRY* was detected in both groups. Group 1 had a significantly lower copy number ($p=0.04$) compared to male control samples. Mean relative copy number *SRY* in the AV+ group: 1.97×10^{-7} (SD 2.91×10^{-7}), AV- group: 2.32×10^{-6} (SD 2.99×10^{-6}), Male control: 1.0×10^{-2} (SD 1.0×10^{-2}), H2O control samples were not detected.

Proportion of SRY present in allotransplant

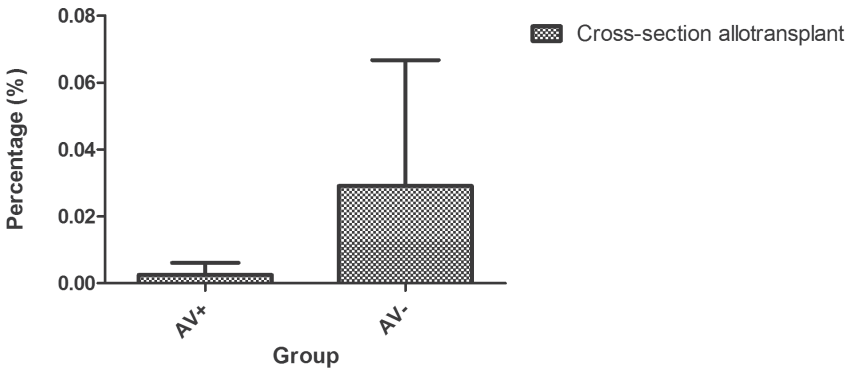


Figure 7: the proportion of SRY present in cross-sections of the allotransplants after 20 weeks. This proportion has been calculated by the formula described in the methods. Where we set the relative copy number of SRY in male control samples as 100%. No significant difference could be found between the groups. The patent AV bundle group had a mean of 1.7×10^{-3} % of SRY (SD: 2.5×10^{-3}), whereas the ligated group had a mean of 1.7×10^{-2} % (SD: 2.4×10^{-2}).

Liver and spleen samples

To evaluate if systemic microchimerism occurred in recipients, we analyzed liver and spleen samples. In gDNA samples, the SRY gene was below the level of detection, indicating that microchimerism in these organs is negligible (Fig. 8).

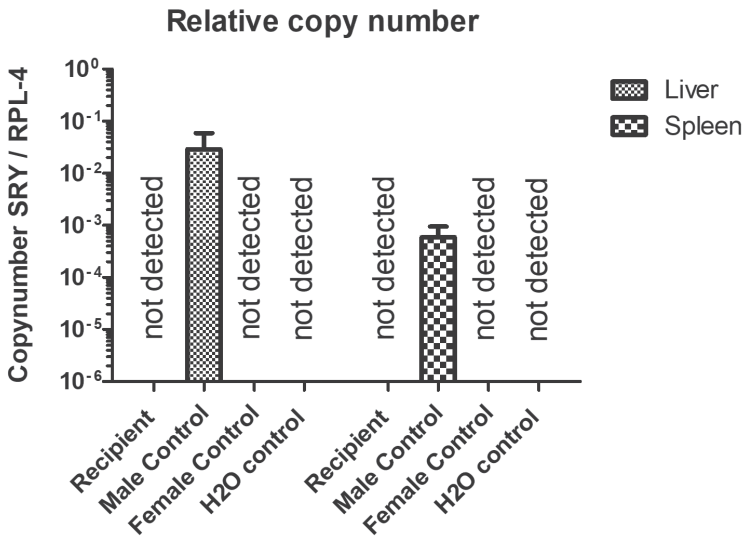


Figure 8: q-PCR analyses of gDNA extracted from pulverized liver and spleen samples. No mixed chimerism was found at 20 weeks after transplantation.

RNA analyses

From the same pulverized bone samples, RNA was extracted as described above and RT-qPCR was performed to assess gender-specific RNAs. We observed that male RNA is still expressed indicating that at least some allogeneic cells remain viable in both groups, with no significant difference between the groups (Fig. 9).

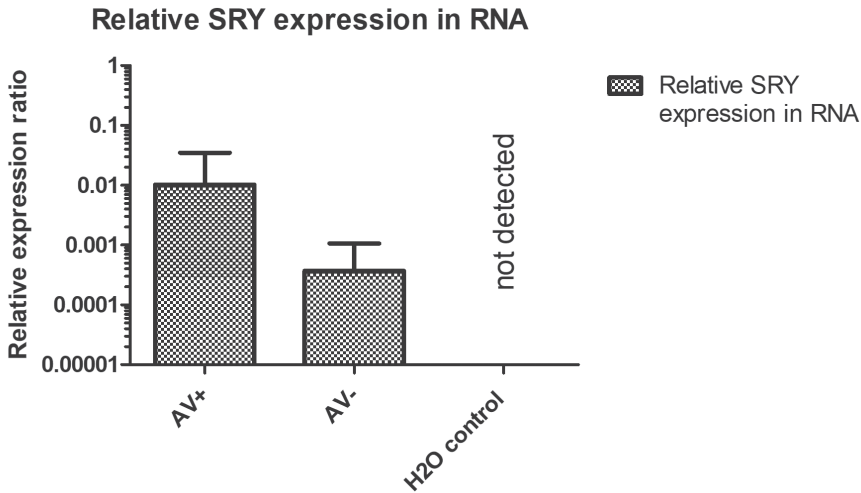


Figure 9: RT-qPCR analyses of RNA extracted from pulverized bone transplant samples. both groups show relative expression of SRY. No significant difference was found between the groups. RPL-4 was used as a housekeeper. Mean relative expression of SRY in the AV+ group: 1.0×10^{-2} (SD 2.0×10^{-2}), AV- group: 1.0×10^{-4} (SD 2.0×10^{-4}), Male control: 2.0×10^{-2} (SD 8.0×10^{-3}), H2O control samples were not detected with qPCR.

Discussion

Reconstruction of large segmental bone defects remains a difficult clinical problem. Current treatment options include cryo-preserved bone allografts and vascularized bone autografts. Allograft segments are readily available and selected to match defect size and shape. With internal fixation, they provide desirable immediate stability. They remain largely necrotic over time and are therefore susceptible to infection, non-union and late stress fracture^[16, 19]. Vascularized bone autografts for large defects are largely limited to the fibula and iliac crest free flaps. They remain viable due to the microsurgical repair of their blood supply. Healing is more assured and rapid than nonviable banked allograft bone. Resistance to infection and the ability to hypertrophy are other desirable qualities.^[20, 21] Donor site morbidity can be significant. The shape of the transferred fibula or iliac crest seldom closely matches the defect morphology. This reduces initial stability, with the risk of early stress-fracture and loss of fixation^[22, 23].

Vascularized composite allotransplantation (VCA) bone represents a potential alternative source of structural bone segments with both the desirable size and shape of banked cryopreserved bone and the many advantages of autogenous bone [24]. Currently, VCAs rely on the use of life-long immunotherapy to maintain vascular pedicle patency and tissue viability. The cost of drugs and monitoring are significant, as are the risks of immunosuppressive drug therapy for a lifetime. Risks include graft-versus-host disease, opportunistic infections, malignancy, metabolic diseases, and end-organ toxicity [25-28].

We have demonstrated bone-only vascularized composite allotransplants to survive long-term, heal and remodel without sustained immunosuppression by surgical implantation of autogenous arteriovenous bundles or fascial flaps within bone. The result is the generation of an autogenous neoangiogenic circulation and subsequent active healing and remodeling of the allotransplant by autogenous circulation-derived cells [11, 29-31]. In this report, a segmental tibial defect model in Yucatan minipigs was developed as a pre-clinical model, to demonstrate similar outcomes and confirm findings obtained in laboratory rats and rabbits.

Movement of cells into the bone (transplant chimerism) and from bone into other tissues (mixed chimerism) has been demonstrated following bone VCA in rat femora, made possible by sex-mismatched organ transplantation [14, 32, 33]. Detection of transplant chimerism from whole segments of VCA bone is of less interest than sampling small areas of bone where active bone remodeling is occurring post-transplant. Laser capture microdissection allows such samples to be obtained and have shown new bone to be primarily autogenous in the rat femoral model [32]. In this porcine study, large structural tibial VCAs used to reconstruct segmental defects demonstrate similar findings.

Lineage studies in calcified tissue are challenging. Decalcification of bone followed by formalin fixation and paraffin embedding (FFPE) resulted in loss of fluorochromes used to label areas of active bone formation and degraded DNA. While frozen bone sections were not similarly affected, they did not adhere as effectively as FFPE specimens to PEN membranes needed for LCM. Reduced adhesion makes capturing very small areas, for example containing a single or a few osteocytes difficult (Fig. 1A). Nevertheless, sampling areas primarily between the double fluorochrome labels demonstrated no remaining allogenic (*SRY*) DNA in seven of eleven samples. This finding serves to confirm that much of the active remodeling and healing occurring in these large bone VCAs is the result of autogenous, likely circulation-derived bone formation. Due to the expense, technical difficulty, and surgical complexity of these large animal experiments, group size and survival period were necessarily limited. Nevertheless, our findings are of importance as the only large animal bone allotransplantation study in the literature. It is fundamentally different from the porcine hindlimb study of Kuo et al. [34], due to the marked differences in antigenicity between bone and the soft tissue components included in their study.

Previously, analysis of areas of new bone formation in rat femur VCAs by laser capture microdissection has shown the majority of osteocytes to be autogenous in origin rather than of allogeneic lineage at 18 weeks post-transplant [32]. These data were obtained by qPCR analysis of the *SRY* gene after female-to-male sex-mismatched bone allotransplants. Although some allogeneic osteocytes may survive due to their relatively sequestered location in lacunae of

calcified tissue ^[35, 36], they are unlikely to be the source of bone remodeling and healing, at least in the laboratory rat model. Analyses of our current large animal bone allotransplantation model demonstrated similar outcomes with respect to areas of new bone formation. Although Group 1 cross-sections contained a lower percentage of extracted male DNA, the RNA extracted from these same pulverized whole bone sections demonstrated a trend towards more metabolic activity. This might be a possible effect of the autogenous angiogenesis. Analyses of RNA confirms the survival of some allogeneic cells in 6 out of 11 samples, although of unknown cell type (Fig. 8).

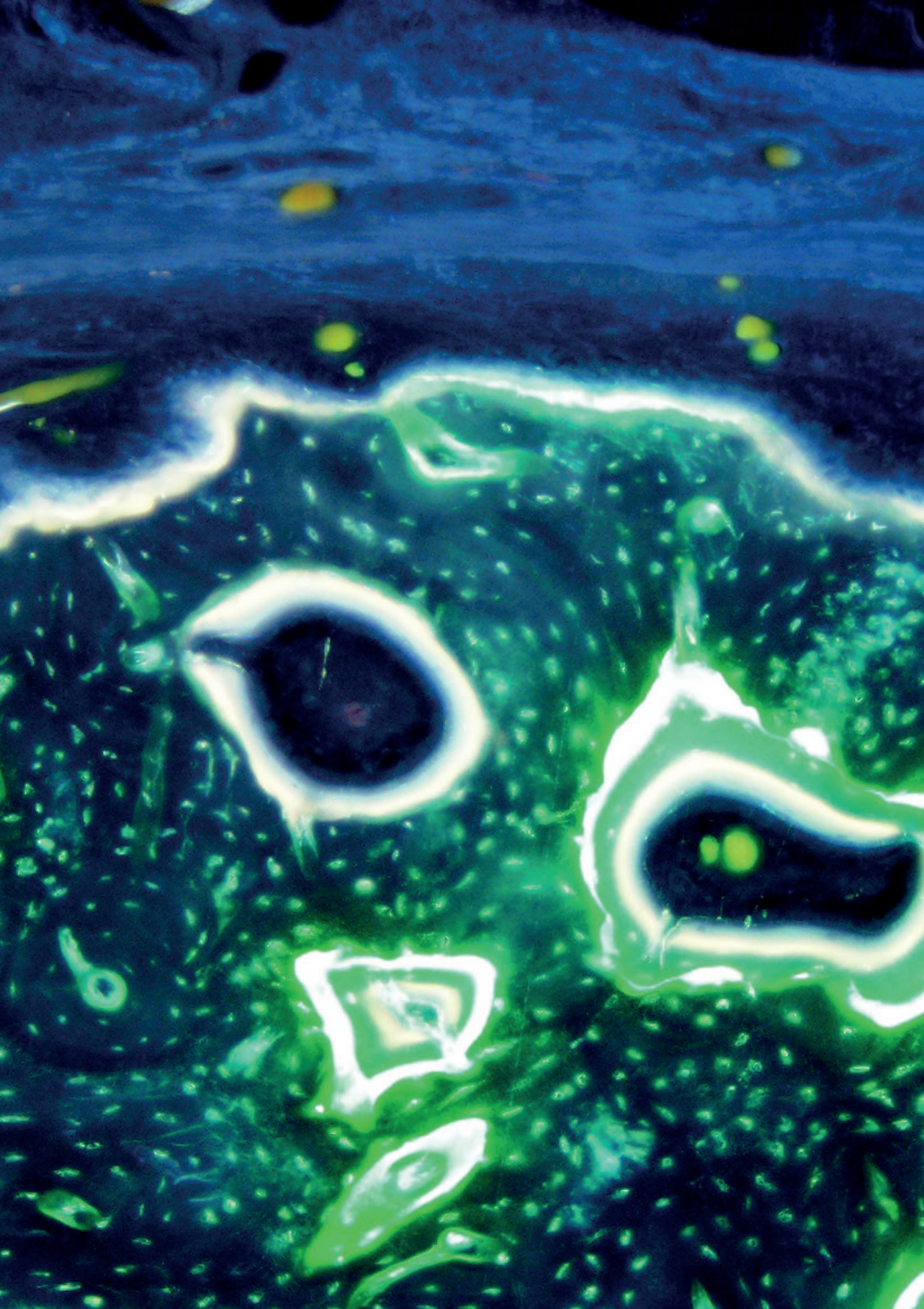
Conclusion

Vascularized composite allotransplantation of bone holds promise for future reconstruction of large segmental bone defects if the need for life-long immune modulation can be obviated. We have demonstrated a unique method to maintain viability, by switching the endosteal circulation of bone allotransplants from its original allogeneic nutrient blood supply to one of autogenous origin. In this report, we studied cell lineage within sex-mismatched bone VCAs by laser capture microdissection and RT-qPCR in a large animal model. Analyses of areas of new bone formation showed significant levels of microchimerism, demonstrating new bone formation to result from the migration of autogenous cells. Some allogeneic male donor cells survived, demonstrated by RNA analyses. No systemic chimerism was found in the liver and spleen.

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7

CHAPTER

**Autogenous arteriovenous
bundle implantation maintains
viability without increased
immune response in large
porcine bone allotransplants**

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Abstract

Background: Transplantation of living allogeneic bone segments may permit reconstruction of large defects, particularly if viability is maintained without immunosuppression. Development of a new autogenous osseous blood supply accomplishes this goal in rodent experimental models. This study evaluates potential systemic and local inflammatory responses to such angiogenesis in a large animal model.

Methods: Vascularized allogeneic tibia segments were transplanted orthotopically into matched tibial defects in Yucatan minipigs. Microvascular anastomoses of bone nutrient artery and vein were supplemented by intramedullary placement of an autogenous arteriovenous (AV) bundle in Group 1. Group 2 served as a no-angiogenesis control. A 3-drug immunosuppression regimen was discontinued after 2 weeks. During the 20-week survival period, periodic leucocyte counts, and inflammatory cytokine levels were measured. Thereafter, osteocyte survival was quantified, and transplant rejection graded by histology and RT-qPCR of immunological markers.

Results: Both groups developed an initial systemic response, resolved after 4-6 weeks. No differences were seen in blood cytokine levels. IL-2 expression was diminished in Group 1 tibiae. As expected, nutrient pedicles had thrombosed without sustained immunosuppression, occluded by intimal hyperplasia. In Group 1, angiogenesis from the autogenous AV-bundle resulted in significantly less osteonecrosis ($p=0.04$) and fibrosis ($p=0.02$) than Group 2 allotransplants.

Conclusion: Systemic immune responses to large bone allotransplants were not increased by generation of an autogenous osseous blood supply within porcine tibial bone allotransplants. Implanted AV bundles diminished inflammation and fibrosis, and improved bone viability when compared to no-angiogenesis controls.

Introduction

Vascularized composite allotransplantation (VCA) is used clinically for reconstruction of complex musculoskeletal tissue loss. The ability to replace 'like with like' offers a potential improvement over conventional methods in some complex reconstructive situations. All VCA procedures currently require life-long immune modulation to maintain viability to prevent acute and chronic rejection. This approach unfortunately may cause organ toxicity, induction of neoplasms and opportunistic infection among other adverse effects as well as considerable effort and expense to monitor and treating rejection over a lifetime. Bone-only VCA for reconstruction of large bone defects has similar potential and limitations, generally for less life-critical indications^[1, 2]. The few reported instances of bone and joint allotransplantation have relied upon drug immunosuppression for tissue survival^[3-5]. At present, this requires multi-drug immunosuppression^[6, 7]. Efforts to induce donor-specific tolerance have not been proven possible^[2, 8].

Alternatively, bone only VCA viability may be preserved by developing a neoangiogenic autogenous blood supply. This effectively switches the circulation that, once developed, will maintain viability without immunosuppression beyond a short initial period. This novel method has proven its potential in multiple studies in rat and rabbit VCA experiments^[9-15]. We have demonstrated extensive angiogenesis from implanted AV bundles or facial flaps, improved bone blood flow, active bone remodeling and healing as well as bone material properties equivalent to free vascularized bone autografts. We have demonstrated that bone survival is not due to donor-specific tolerance nor immune deficiency. Transplant survival is facilitated by transplant chimerism, due to repopulation by recipient-derived osteocytes from the new autogenous circulation^[16-18].

Recently, we have developed a large porcine tibial orthotopic transplant model to demonstrate similar angiogenesis, healing and limb function as pre-clinical verification^[19, 20]. The well-studied pig immunology further permitted this current study of systemic and local immune responses to autogenous angiogenesis within VCA bone segments.

Methods

Experimental design

This study was approved by the Institutional Animal Care and Use Committee and was performed according to established National Institutes of Health guidelines. Orthotopic vascularized bone allotransplantation was performed in fourteen Yucatan miniature swine divided into 2 equal groups (Sinclair Bioresources, LLC). An additional 7 animals served as donors for two tibiae each, matched by age (mean 5.8 months), size (15-35kg) and blood type (type A). Donor and recipient animals were mismatched by pre-operative swine leukocyte antigen (SLA) haplotyping. Pre-operative DNA sequence haplotyping ensured a mismatch of five to ten class I and II SLA markers. Microsurgical tibial transplantation was performed in all animals as previously described [20], transplanting a 3.5 cm tibial segment orthotopically into a matched defect. Rigid internal fixation was made with dual locked plates. The nutrient blood supply was restored with microsurgical anastomoses of both artery and vein. Simultaneously, an autogenous cranial tibial arteriovenous (AV) bundle was raised and placed within the VCA medullary canal in Group 1 (Fig. 1). It was placed but ligated as a no-angiogenesis control in Group 2. A central venous line was maintained for intravenous drug administration and blood draws during the first two weeks. Immunosuppressive therapy was given for a period of 14 days, including Tacrolimus 0.6-1.5mg/kg (Sandoz Inc. Princeton, NJ), Mycophenolate Mofetil 30-60mg/kg (Mylan Institutional Inc., Rockford, IL) and Methylprednisolone sodium succinate (Pfizer Inc., New York, NY). Tacrolimus and Mycophenolate were administered orally and Methylprednisolone intravenously. Doses were adjusted as needed to maintain therapeutic levels (Tacrolimus: 5.0-15.0 ng/ml, Mycophenolate 1.0-3.5 mcg/ml), monitored by blood draws taken every other day. The methylprednisolone initial dose was 500 mg, tapered over 2 weeks (100-12.5mg/day). Antibiotic prophylaxis was given with Enrofloxacin 7.5mg/kg IM (Baytril, Bayer Healthcare LLC, Shawnee Mission, KS) and Ceftiofur 5mg/kg IM (Excede, Pfizer Inc, New York, NY). Full weight-bearing was allowed immediately. Pain was monitored daily and treated with appropriate analgesics (Carprofen an Buprenorphine). The animals were individually housed and received standard pig feed and water ad libitum. At 2, 4, 6, 10 and 20 weeks, the pigs were anesthetized with Tiletamine HCL + Zolazepam HCL 5 mg/kg IM (Telazol, Zoetis Inc, Kalamazoo, MI.) and Xylazine 2 mg/kg IM (Xylamed, Bimeda-MTC, Cambridge ON, Canada) for wound inspection and blood draws. Drug immunotherapy was stopped, and the central venous catheter removed on postoperative day 14. The survival period was 20 weeks.

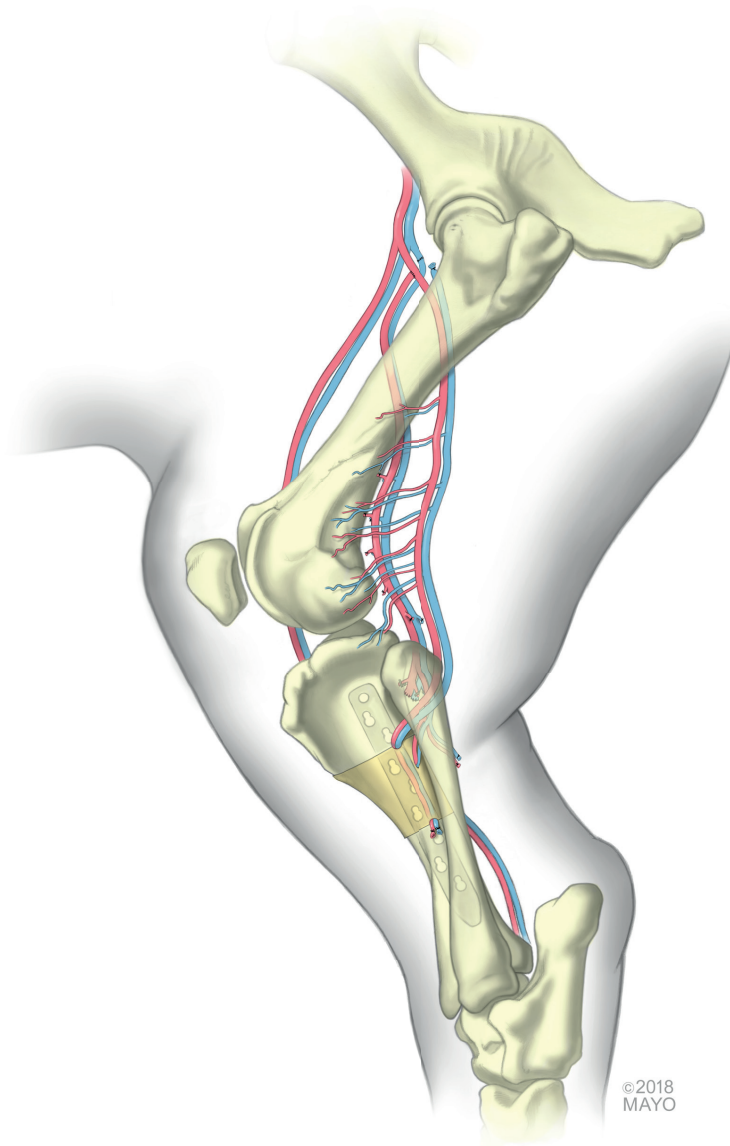


Figure 1: illustration of the reconstruction in a porcine hind-limb model. The vascularized segment is microsurgically transplanted into a created defect in the recipient. Ridged internal fixation is achieved with locking plated. The allogenic micro anastomosis is made onto the femoral vessels of the recipient. Simultaneously the autogenic arteriovenous bundle is implanted into the medullary canal of the allotransplant.

Systemic inflammatory response

To evaluate the systemic immune response, we obtained blood at baseline (before surgery) and at 1, 2, 4, 6, 10 and 20 weeks after surgery. A total of 10ml blood was obtained with heparin (50USP per 10ml of blood). Five milliliters were used for hematology tests, including differential leucocyte counts. The remainder was centrifuged for 10 minutes at 3000 RPM at 5°C. The supernatant was transferred into a clean 1.5ml microtube and stored at -80°C. Cytokine analyses were then performed using a Milliplex porcine cytokine/chemokine magnetic bead panel (Millipore Sigma, Cat. No PCYTMG-23K-13 PX, Darmstadt, Germany). This panel enabled quantification of thirteen different cytokines. The panel allowed measurement of: IL-1A, IL-1B, TNF-A, INF-G, IL- 2, IL-4, IL-6, IL-8, IL-10, IL12, IL-18 and GM-CSF, especially designed for porcine cytokines. This multiplex analysis was performed by the Immunochemical Core laboratory at our institution following the manufacturer's protocol.

Sacrifice procedure

At 20 weeks, all animals were anesthetized with Tiletamine HCL + Zolazepam HCL. Thereafter, the animals were euthanized as recommended by the Panel on Euthanasia of the American Veterinary Medical Association with Pentobarbital Sodium (Vortech Dearborn MI, 0.22 ml/kg IV). The entire tibia including the allotransplant was harvested under sterile conditions, removing the internal fixation. The allotransplant was divided using a cooled oscillating saw, including a 2mm section snap-frozen in liquid nitrogen and stored at -80°C reserved for PCR analyses, and a 5mm bone section reserved for histology, fixed in 10% buffered formalin for 48h. The 5mm bone section was embedded in methyl methacrylate and sectioned into 15µm-thick sections using a diamond band saw (Exakt Technologies Inc., Oklahoma City, OK) and stained with hematoxylin/eosin(H&E).

Transplant Pedicle

The allogenic vascular pedicle and the site of the microsurgical arterial and venous anastomoses were identified, removed, fixed in 10% buffered formalin for 48h. The vascular pedicle was embedded in paraffin, sectioned in 5µm-thick sections, deparaffinized and stained with an Elastica-van Gieson stain.

Inflammatory response of allotransplant

In order to evaluate the local inflammatory response of the allotransplant, we used an existing histologic score of bone inflammation (the Histologic Osteomyelitis Evaluation Score or HOES) for the purpose of analyzing rejection of bone allotransplants [21]. We modified the HOES score by examining the endosteal surface and intramedullary space on the H&E stained bone sections for osteonecrosis, soft tissue fibrosis and inflammatory infiltrate. Each factor was scored on a 0-3-point scale: non-existent= 0, mild= 1, moderate= 2, or severe= 3 in which 1= <25%, 2= 25-50%, 3= >75% of the field of interest. A total score of 9 points could be received for each sample by scoring osteonecrosis, soft-tissue fibrosis, and inflammatory infiltrate. We examined 6 fields selected at random in each sample. In order to quantify osteonecrosis more accurately, we used a bone image analyses system to measure osteocyte counts (OsteoMeasure; OsteoMetrics, Atlanta, GA). The extend of osteonecrosis is defined by the percentage of empty lacunae per field of interest (FOI).

Immunogenicity of allotransplant by quantitative real-time-PCR (RT-qPCR)

We measured levels of inflammatory cytokines within bone samples harvested at the 20-week survival time by RT-qPCR. The cytokines were selected by relevance to allotransplantation, and included *TNF*, *IL2*, *IL6*, *IL8*, *INFG*, *CD4*, *CD8a*, and *CD28*. *GAPDH* served as a reference gene (Table 1). The 2mm, previously flash-frozen bone sections were cleared of soft tissue and pulverized in liquid nitrogen using the A11 basic analytical mill (IKA-Werke GmbH & Co. KG, Germany). RNA was extracted from the pulverized bone with the PureLink RNA mini kit, TRIzol reagent and on-column Pure link DNase treatment (Thermo Fisher Scientific, Cat no. 12813018A, 12034977, 12185-010, Carlsbad, CA). RNA purity and quantification were performed on a Nano-drop Spectrometer (ThermoScientific Nano-drop Technologies, Wilmington, DE). The absence of RNA degradation was confirmed by gel electrophoreses before the RNA was converted to cDNA. cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA) following the protocol of the manufacturer with 200 ng of RNA. Quantitative real-time PCR (RT-qPCR) was performed to quantify the expression of target genes with iQ SYBR green Supermix and the CFX384 Real-Time detection system (Bio-Rad Hercules, CA). Transcript quantity measurements were normalized to *GAPDH*, and gene expression levels quantified using the $2^{-(\Delta\Delta CT)}$ method [22]. Primer sequences are given in table 1 (ThermoScientific, Invitrogen, Wilmington, DE). Real-time PCR was followed by melt curve analyses with the following conditions: 15min denaturation at 95°C for one cycle, 20s of denaturation at 95°C, 35s of annealing and extension at 72°C for 51 cycles followed by generation of a melting curve. Melt curves were performed from 60 °C to 95°C with an increment of 0.5°C.

CHAPTER 7

Table 1: primer sequence and name for each gene of interest

Gene	Full name	Sequence
Immunology markers		
<i>TNF</i>	Tumor Necrosis Factor alpha	5'-3': CCACCAACGTTTTCTCACT 3'-5': CCAAAATAGACCTGCCAGA
<i>IL2</i>	Inter Leukine 2	5'-3': CAAACGGTGACCTACTTCA 3'-5': CCTGCTTGGCATGTAAAAT
<i>IL6</i>	Inter Leukine 6	5'-3': ATGGCAGAAAAAGACGGATG 3'-5': GTGGTGGCTTTGTCTGGATT
<i>IL8</i>	Inter Leukine 8	5'-3': TGGCAGTTTTCTGCTTTCT 3'-5': CAGTGGGGTCCACTCTCAAT
<i>IFNG</i>	Interferon gamma	5'-3': TTCAGCTTTGCGTGACTTTG 3'-5': TCCTTTGAATGGCCTGGTTA
<i>CD4</i>	Cluster Differentiation 4	5'-3': GCTGGGGAACCAGAGTATGA 3'-5': AGAACCCAGCGAGAAACAGA
<i>CD8a</i>	Cluster Differentiation 8a	5'-3': TGCACTCCAACACTGACA 3'-5': TGTCATTGGCCTTGTAACCA
<i>CD28</i>	Cluster differentiation 28	5'-3': TCGGCCTCTGAGTCTTCTA 3'-5': AGTCACGTGCTGGTGCATAG
Housekeeper		
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	5'-3': ACACTCACTCTTCTACCTTTG 3'-5': CAAATTCATTGCTGACCAG

Statistical analyses

Since the data was collected from a low sample size (N=14), a non-parametrical test (Wilcoxon rank sum test) was used to detect a difference between the two groups (allografts with Patent AV bundle versus allografts with ligated AV bundle) as well as differences within each animal. All statistical tests were two-sided and differences were considered significant for p-values of <0.05. Statistical analyses were performed using JMP Pro 13.0.0 (SAS Institute Inc.) and GraphPad Prism 5.03 for illustrations (GraphPad Software, La Jolla, CA). Statistical analysis was supported by the Center for Translational Sciences Activities (CTSA) at our institution.

Results

All animals were fully weight-bearing at an average of 4 days after transplantation and gained an average of 20.0kg in weight during the survival period. During the 2 weeks of initial immunosuppression, drug levels remained in therapeutic range for all animals. In the first 2-4 weeks, six animals developed seromas in the inguinal area, which resolved spontaneously. Four animals were excluded from analyses for either deep infection, unrecognized pregnancy, uncontrollable seizures or breach of bone harvest technique. This left 5 animals in each group for analysis.

Systemic response to allotransplantation

We determined total white blood cells (WBC), lymphocytes, eosinophils, monocytes, neutrophils and red blood cell (RBC) counts to assess the systemic immunological state of the pigs. Pre-operative cell counts were obtained pre-operative, and 1, 2, 4, 6, 10, and 20 weeks after transplantation. Pre-operative measurements did not show a statistical difference between the two intervention groups. Immunosuppression was started intra-operatively. We observed an increase of total white blood cell counts, as well as lymphocyte, eosinophil, and neutrophil levels during the first postoperative week in both groups. White blood cells returned to baseline levels one week later. Lymphocyte, eosinophil, and neutrophil levels also decreased after the first week but did not directly return to pre-operative levels in either group (Fig. 2). The decrease in blood cell counts after one week is consistent with expected effect of drug immunosuppression.

Two weeks after the transplantation, immunosuppressive therapy was stopped, and the central line surgically removed. We observed a slight increase of total WBC and neutrophil counts after this second intervention. None of the animals showed clinical signs of infection. Seromas at the surgical site were common and resolved spontaneously.

Four weeks postoperatively, eosinophil levels were significantly elevated in the ligated AV-bundle group ($p=0.03$). Between the 4th and 6th week the total WBC, eosinophil and neutrophil levels decreased, and remained stable thereafter. Total WBCs, neutrophils, eosinophils, lymphocytes, and monocytes returned to pre-operative levels from the 6th week until the end of the survival period (Fig. 2).

Additionally, we quantified IL1A, IL1B, TNF, INFG, IL2, IL4, IL6, IL8, IL10, IL12, IL18 and GM-CSF/CSF2 levels in peripheral blood at the same periods (Fig. 3). In both groups, cytokine levels increased in the first week. All cytokine levels decreased after the first week and returned to pre-operative levels (not detectable levels) between the 4th and 6th week and remained stable thereafter (Fig. 3). No differences in systemic immune response were found between the groups for any cytokine. Taken together, the analyses of total blood cell counts and cytokine measurements did not demonstrate substantial immune responses to the allotransplant after 4-6 weeks.

CHAPTER 7

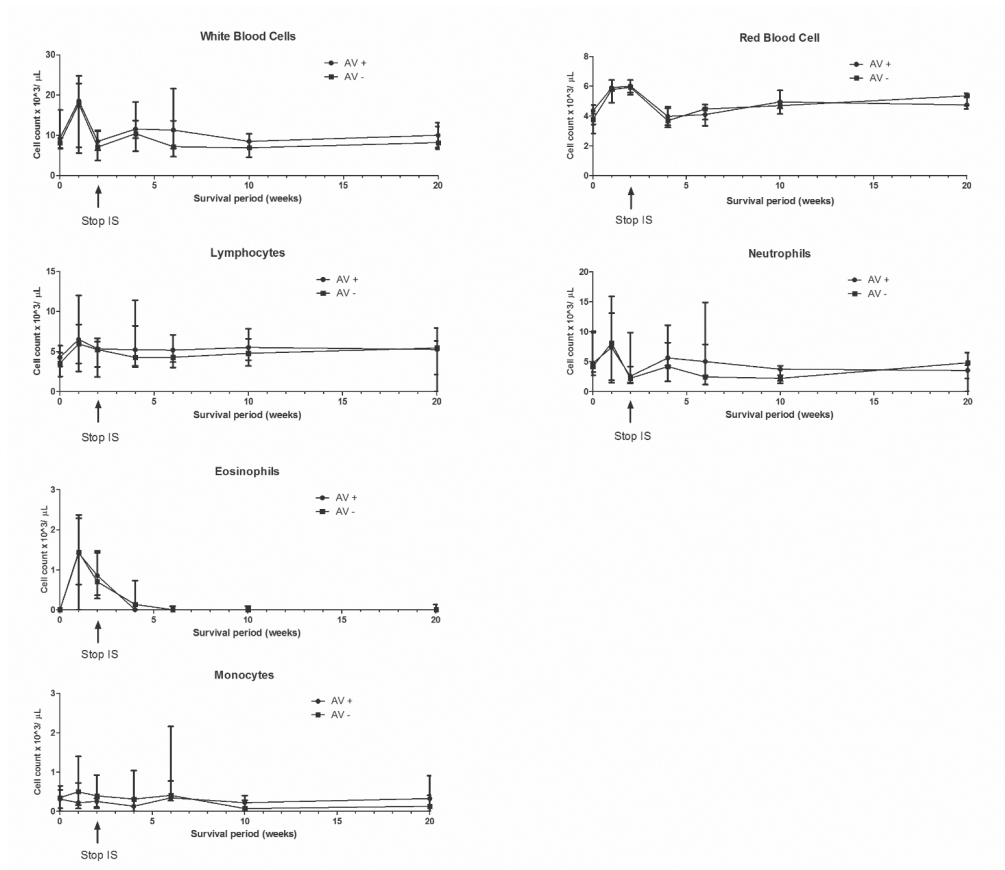
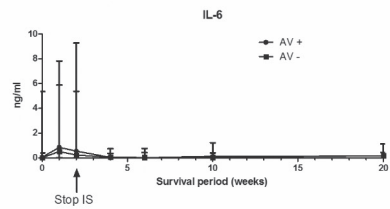
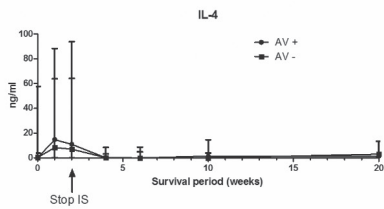
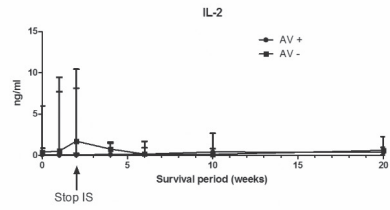
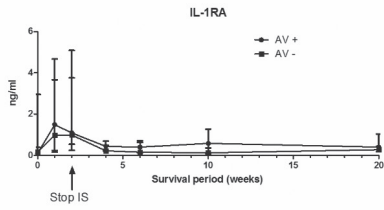
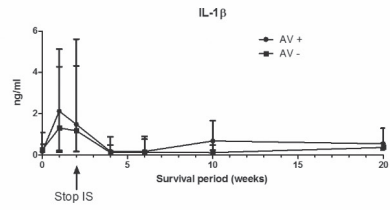
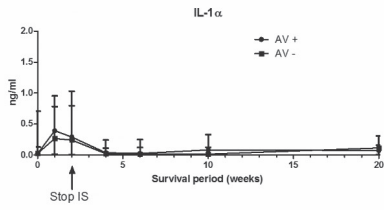
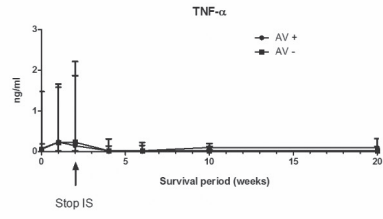
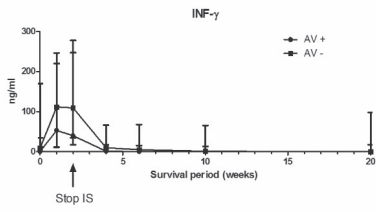


Figure 2: Blood cell counts pre-operative (0), and measured at 1, 2, 4, 6, 10 and 20 weeks after transplantation. AV+ = group 1 with the patent arteriovenous bundle, AV- = group 2 with a ligated arteriovenous bundle.



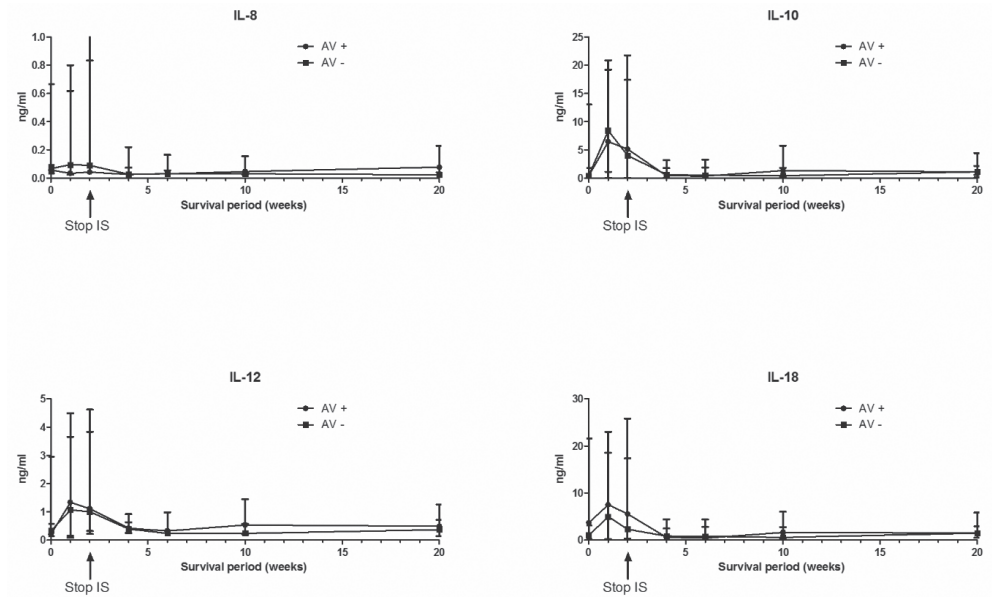


Figure 3: Blood serum cytokine levels pre-operative (0), and measured at 1, 2, 4, 6, 10 and 20 weeks after transplantation. AV+ = group 1 with the patent arteriovenous bundle, AV- =group 2 with a ligated arteriovenous bundle.

Vascular changes

Twenty weeks after transplantation all allogeneic vascular pedicles had thrombosed. The allogeneic nutrient artery supplying the bone failed due to intimal hyperplasia a finding pathognomonic of rejection (Fig. 4) [1, 23]. Allogeneic periosteal arteries also demonstrated similar changes, consistent with rejection (Fig. 5C). In contrast, the autologous implanted AV-bundle arterial walls remained histologically normal and the vessels patent in group 1 (Fig. 5A). Hence, allogeneic vascular properties in and near the transplant are all consistent with host graft rejection. While, the autogenous AV bundle implantation remains patent without signs of rejection by the allotransplant.



Figure 4: Histology of the allogenic vascular pedicle: thrombosed vascular pedicle, hypertrophic intima with an intact internal elastic lamina (Elastica-Van Gieson stain).

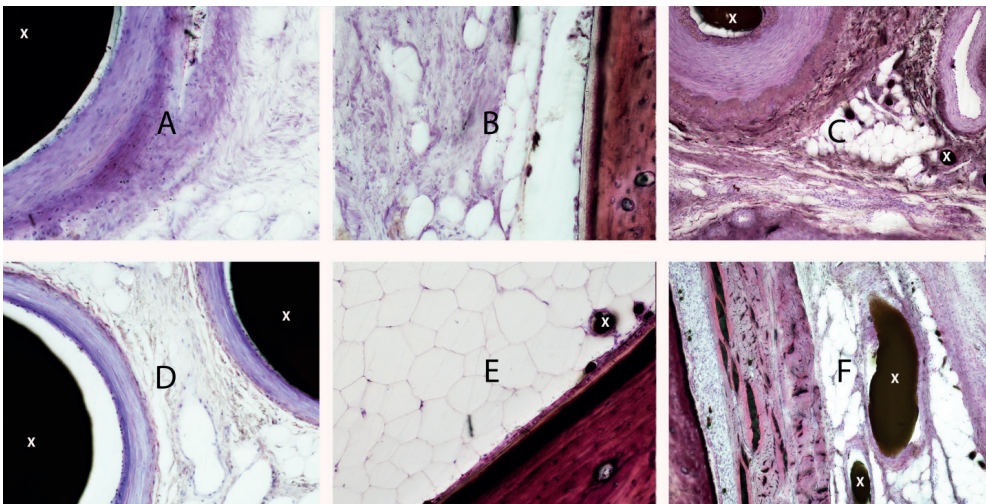


Figure 5: Histology of different soft tissues 20 weeks after transplantation (stained by hematoxylin and eosin): the arteriovenous(AV) bundle within the allotransplant showing a normal and patent arterial wall (A) with some fibrotic changes within the intramedullary canal of the allotransplant (B), compared to a normal intramedullary artery (D) and canal (E) of the contralateral tibia. (D). In the periosteal soft tissue we found arteries showing signs of intima thickening and fibrosis of the allotransplant (C) compared to the normal contralateral side (D) all the black area marked with x is a contrast agent Microfill indicating the patency of the vessels.

Inflammatory response of allotransplant

Histology bone slides were scored to quantify the inflammatory response of the allotransplant. We observed significantly ($p=0.04$) less osteonecrosis on the endosteal surface of the allotransplant in the patent AV bundle group (Fig. 6, Table 2). In addition, we observed significantly less ($p=0.02$) intramedullary fibrosis and fat necrosis in the patent AV bundle group (Table 2). Both allotransplant groups showed mild patchy infiltration of lymphocytes in the intramedullary space. Using the modified HOES-score, we found the total inflammatory score of bone allotransplants on the endosteal surface to be significantly reduced ($p=0.04$) in the patent AV bundle group. Thus, significant reduced total inflammatory scores were found due to AV bundle implantation.

Table 2: Inflammatory score bone allotransplants on the endosteal surface (H&E slides)

Group	Percentage of empty endosteal lacunae (%)	Osteocyte Score	Intramedullary fibrosis	Infiltration of lymphocytes	Total score (max=9)
Patent AV-bundle	11.7 (9.48-12.81)	1 (0-1)	1 (1-1.5)	1 (0.5-1)	3 (2.0-3.0)
Ligated AV-bundle	21.65 (13.01-29.64)	1 (1-1.25)	3 (1.75-3)	1 (0.75-1)	5 (3.5-5.25)
P-value	0.04*	0.07	0.02*	0.891	0.04*

Score system: 0= None, 1= mild (<25% of FOI), 2= moderate (25-75% of FOI)

3= severe (>75% of FOI)

Values are given in mean and SD, field of Interest (FOI),* significant

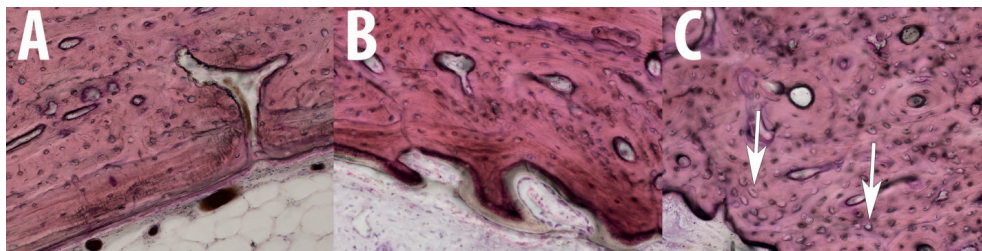


Figure 6 : Histology of bone (Hematoxylin and Eosin staining) showing the normal (contralateral) endosteal morphology of bone (A) compared to the endosteal morphology of the allotransplants (B/C). The lacunae of the allotransplants with a patent arteriovenous (AV) bundle (B) where significantly more occupied by osteocytes compared to the allotransplant in the ligated AV bundle group (C). Demonstrated by the white arrows in C pointing at some of the empty endosteal lacunae.

RT-qPCR analyses

Analyses of a panel of immunoresponsive mRNA biomarkers in the allotransplant by RT-qPCR showed no statistically significant differences in expression between the two intervention groups compared to the normal (contra-lateral) bone for any of the genes we tested (Fig. 7). Interestingly we did find a statistically significant reduced ($p=0.015$) expression of IL2 in the patent AV-bundle group (group 1) compared to group 2. The latter result indicates that inflammation was attenuated in this treatment group. Thus, no significant ongoing immune response was observed within the allotransplant 20 weeks after transplantation.

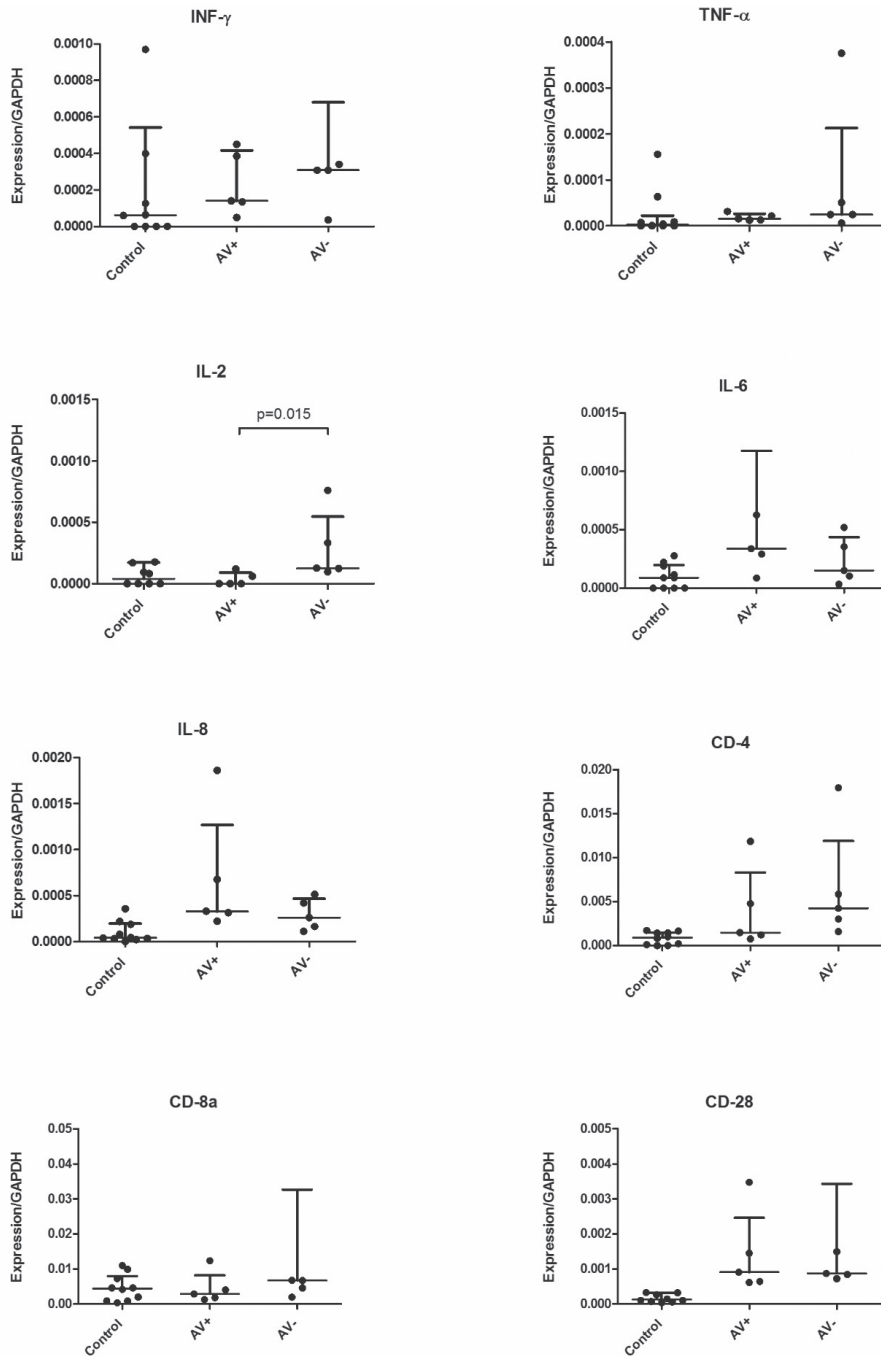


Figure 7: Relative expression of INFG, TNF, IL2, IL6, IL8, CD4, CD8a, CD28 measured in the allotransplants in the patent arteriovenous (AV+) bundle group, ligated AV bundle group (AV-), and normal (contralateral) bone as control. Relative expression was measured by amplification of the target genes in RNA by RT-qPCR.

Discussion

Large segmental bone defects are often reconstructed today with structural cryopreserved allografts, and vascularized bone autografts, used alone or in combination. Allografts provide immediate strength and stability but remain largely non-viable. Significant rates of non-union, infection and late stress-fracture are the result [24]. Vascularized bone autografts may hypertrophy in response to mechanical load, heal more readily and resist infection [25]. They are generally poorly matched to defect size and shape and result in some donor site morbidity [26]. Bone-only VCAs may in the future offer the best of both methods: with immediate stability and strength if matched to the defect, yet with similar healing and remodeling potential as vascularized bone autografts. Few have been used clinically, as VCAs require life-long immunosuppression, with the previously-described risks, periodic evaluation and expense. The novel method we have described permits bone-only VCA survival without prolonged drug therapy.

Porcine animal models have distinct advantages for allogenic tissue transplantation research in order to retrieve pre-clinical translatable results. Their size, anatomy, physiology and immunology are well known and comparable to man. Furthermore, as blood type and swine leukocyte antigen (SLA) haplotypes have been well studied [27, 28]. Experimental transplantation with manipulation of these variables in these large animals provides better insight into potential clinical utility than studies of smaller rodents [20, 29]. Long-term monitoring of systemic immune responses after transplantation can be accomplished by hematology studies and cytokine detection. Immunosuppressive drug levels are performed as routine clinical tests.

The immune response within the bone allotransplant at 20 weeks was also analyzed by RT-qPCR. Segments of each transplanted bone were pulverized, and specific messenger RNA sequences were used to investigate the specific nature of the immunologic activity. Previous investigators have found the need for decalcification to render other methods such as in-situ hybridization or immunohistochemical staining unreliable. A mild patchy lymphocytic infiltration was found in both groups, most likely the result of an inflammatory response to the allogeneic material. Quantitative RT-qPCR analyses of messenger RNA further identified the presence of immunologic markers within the allotransplant, although not significantly elevated compared to normal bone 20 weeks after transplantation. These results are important to better understand the immune status of the allotransplant over time.

Vascularized bone and joint allotransplantation has seldom been performed clinically. The largest case series is that of Hofmann, who has performed three vascularized femoral diaphysis and five vascularized whole knee joint transplantations [30, 31]. All patients were matched for bloodtype, no attempt was made to match HLA markers. Maintenance immunosuppression varied in type and length, and included cyclosporin A, azathioprine, anti-thymocyte globulin, and methylprednisolone. Rejection of vascular tissue has been well understood from solid-organ transplantation literature, when this rejection occurs it leads to intima hypertrophy. This type of vasculopathy can lead to transplant ischemia and eventual loss of the allotransplant [23]. Eventually, all of the complete knee joint transplantation cases have failed due to infection, rejection or chronic allotransplant vasculopathy [4, 23, 30-32]. The two of the vascularized femoral diaphysis failed,

one each due to infection and rejection ^[30]. Neither prolonged immunosuppression nor treatment of an acute rejection episodes with drug immunosuppression proved successful. Thus, a different approach is necessary.

In our experimental study, all microsurgically repaired bone vascular pedicles developed intimal hypertrophy and subsequently thrombosed 4-6 weeks after transplantation ^[33]. These changes were as expected after cessation of a 2-week period of immunosuppression and are consistent with vascular rejection ^[23]. Importantly, however, we were able to maintain bone viability despite rejection, made possible by the novel technique of autogenous neo-angiogenesis from an implanted autogenous AV-bundle. This success in a large animal model suggests possible future use in clinical practice.

Conclusion

Bone-only VCAs treated with short-term triple drug immunosuppression and intramedullary implantation of an arteriovenous (AV) bundle demonstrate improved osteocyte scores, diminished fibrosis and less inflammatory change than no-angiogenesis controls. Thrombosis of the allogeneic nutrient artery results from intimal hypertrophy, expected without immune modulation. Necrosis of the bone does not occur, due to development of a neoangiogenic autogenous blood supply during the initial period of drug therapy. In this study, there were no adverse systemic inflammatory effects of this process, studied by periodic peripheral blood cytokine determinations during a 20-week survival period. The bone allotransplants themselves demonstrated less inflammation and improved viability than no-angiogenesis controls, provided by the addition of an autogenous AV bundle. This novel approach to bone allotransplantation may have significant advantages over conventional methods in bone-only allotransplantation.

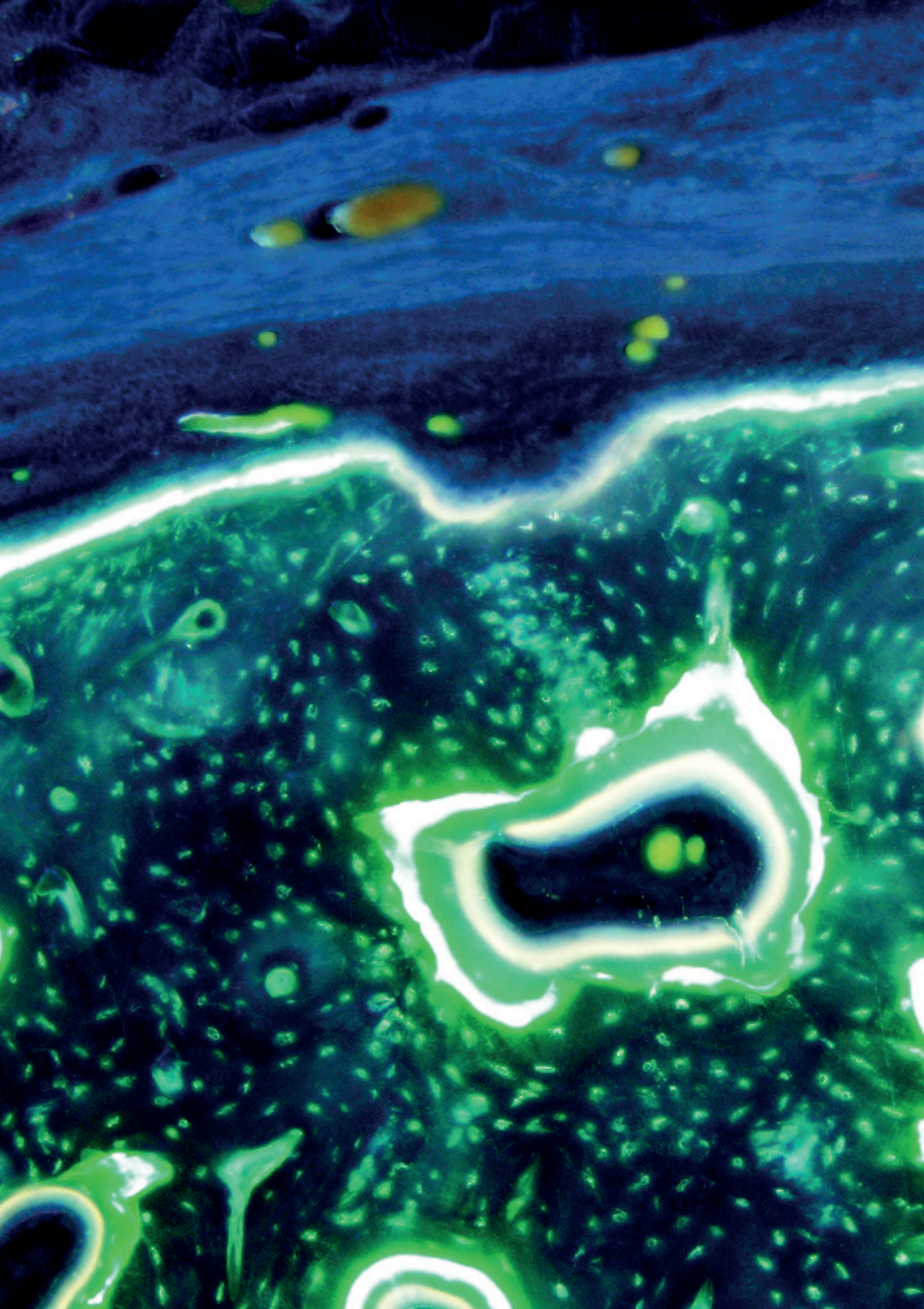
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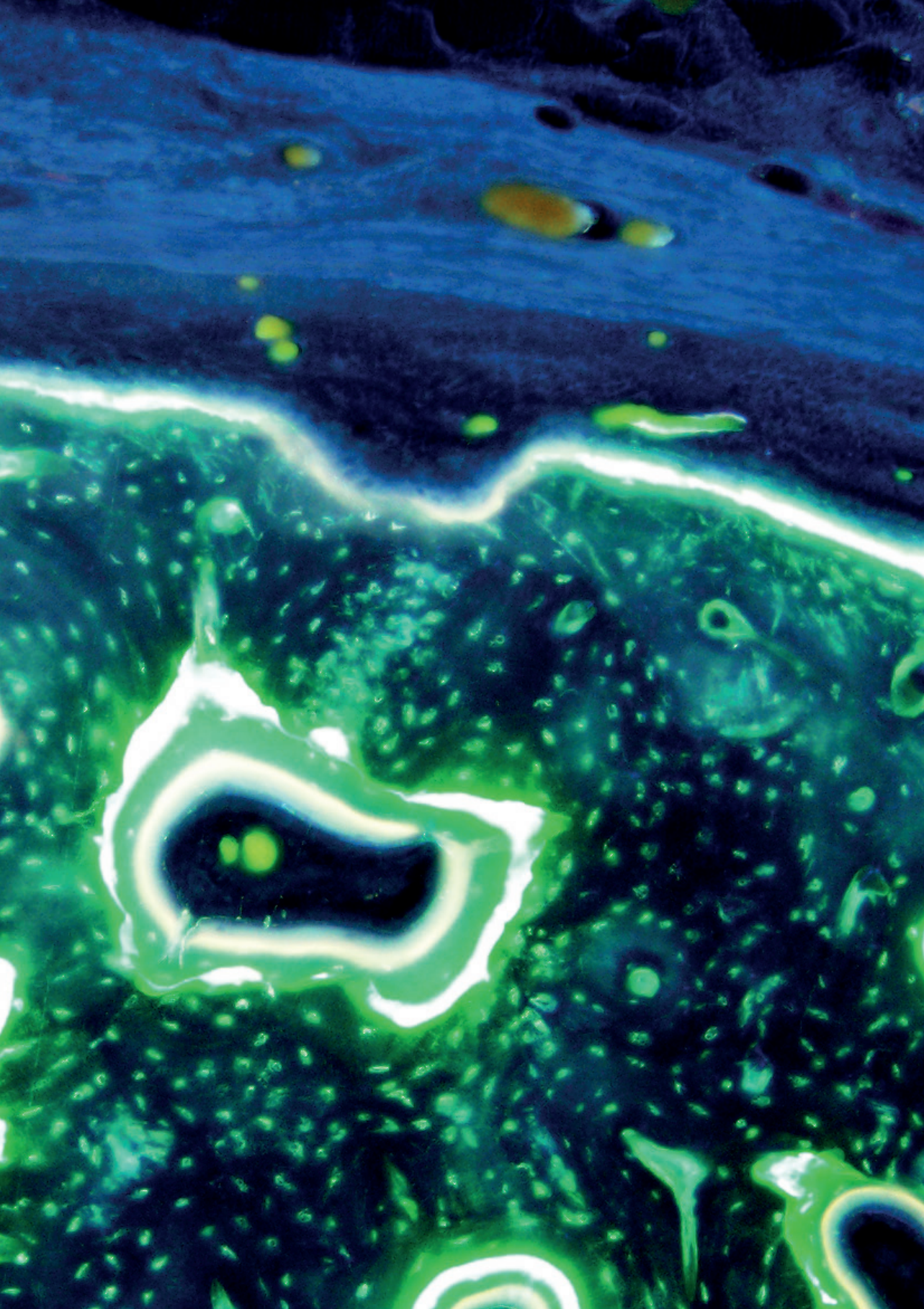
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PART

3





8

CHAPTER

A new porcine vascularized knee allotransplantation model: anatomy, surgical technique, and improvements

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Abstract

Clinical attempts of complete joint allotransplantation in the past have generally resulted in rejection and graft failure. The necessity to better understand the (patho) physiology of knee allotransplants in a pre-clinical model is crucial in order to study the use of vascularized joint allotransplantation for the reconstruction of large joint defects. In this paper, we describe a novel large animal model to study orthotopic vascularized whole knee joint allotransplantation with short-term immunosuppression and autologous revascularization. We performed an anatomy study and feasibility study on allotransplantation of a vascularized whole knee joint with autologous arteriovenous bundle implantation covered by a pedicled gracilis muscle flap in three outbred farm pigs. All animals received two weeks of multi-drug immunosuppressive therapy. All animals had to be sacrificed two weeks after transplantation due to the development of infected seromas. The radiographic and histologic evaluation showed that the allotransplants were viable at the time of sacrifice, but infected due to complications. We have demonstrated an experimental large animal model of knee joint allotransplantation. Despite best efforts, all experimental animals developed wound infections despite pedicled muscle flap coverage and wound closure and protection methods used in the same animals successfully for tibial transplants in the past. The complexity, expense and complications make investigation of vascularized whole knee transplantation in a large animal experimental model challenging. Eventual success with modified postoperative care would permit a better understanding of large joint transplantation for possible future clinical use.

Introduction

Management of extensive destruction of the knee joint after infection, trauma, or primary tumors are a difficult problem. In worst-case scenarios, there is a significant loss of bone, cartilage, soft tissue, and an extensor mechanism defect. In these cases, treatment options are either to perform an arthrodesis, amputation or reconstruction ^[1]. Limb-salvage with joint reconstruction provides a better functional result, an arthrodesis, stability at the expense of motion, and amputation, loss of the limb and function without prosthetic fitting.

Current reconstructive options involve the use of prosthetics, cryopreserved allografts, bone transport, alone or in combination. All reconstructive options are associated with significant complication and failure rates ^[2,3]. Allotransplantation of knee joints is a relative novel approach to reconstruct large defects of the knee. Vascularized composite allotransplantation of bone and joints has been occasionally performed ^[4]. After some initial success, subsequent longer-term evaluation of human knee joint allotransplantation demonstrates all to have failed over time. Due to rejection with vascular thrombosis and tissue loss, or infection ^[1, 5-11]. Experimental models of vascularized knee joint transplantation have been described since 1968 in rodent, lagomorph, feline, and canine ^[5]. The effect of immunosuppression on vascularized knee allografts has been well described in dogs, demonstrating sustained immunosuppressive therapy to positively effect transplant survival ^[12]. Life-long drug therapy is clinically associated with the risk of opportunistic infections, diabetes, hypertension and neoplasms, with costs of drugs and monitoring causing a financial burden ^[1, 3]. The incidence of neoplasms induced by immunosuppressive therapy is estimated to be 4-18% ^[13]. Joint allotransplantation in a rabbit model using short-term immunosuppression and surgically induced neo-angiogenesis resulted in improved transplant viability, bone healing, and bone properties. Flexion contractures, due in part to the hyperflexed resting stance of the rabbit knee caused functional impairment ^[14, 15]. An animal joint transplant model better simulating the human knee function would allow a thorough investigation of joint allotransplant feasibility, including bone healing, joint properties, tissue perfusion, as well as systemic and local immune responses.

In this feasibility study, we developed a technique permitting whole knee joint allotransplantation in pigs. The method included use of rigid intramedullary locked nail fixation of the femur and tibial segments, microsurgical reconstruction of the transplanted joint blood supply, and angiogenesis from implanted autogenous arteriovenous bundles. The latter, described for bone-only allotransplantation, enables cessation of immunosuppression after development of a robust autogenous blood supply.

Materials and Methods

A series of preliminary anatomical studies in porcine cadavers were performed to define the vascular anatomy of the knee blood supply in the pig, and develop techniques to harvest the allotransplant, microsurgically restore blood flow, stably internally fix the femoral and tibial skeleton, implant autogenous vessels into femur and tibia, and provide adequate soft tissue coverage of the reconstruction. Subsequently, three sex-mismatched vascularized whole knee joint allotransplantations with autogenous AV-bundle implantation and muscle coverage were performed with a 10-week survival period.

Anatomic study

Six fresh porcine cadaveric hind limbs were obtained from three outbred pigs. The animals were a mean 25 kg of weight. The hind limbs were dissected to define the vascular anatomy of the knee and surrounding soft tissues. The femoral artery was cannulated, and the limb flushed with heparinized saline 10000 USP/L (0.9% Sodium Chloride, Baxter Healthcare Corporation, Deerfield, IL) (Heparin Sodium Injection, Fresenius Kabi LLC, Lake Zurich, IL). It was next injected with Ward's red latex (Ward's, Rochester, NY). After curing overnight, the lower limbs were dissected and vessel topography relevant for vascularized whole joint allotransplantation defined, including the vascular supply to the gracilis muscle for wound coverage use. Rigid internal fixation of whole knee joints knee has been described in humans with intramedullary nail fixation [1, 5, 7]. We tested and developed the means to use veterinary locked intramedullary nails for both femoral and tibial fixation in these hind limbs. We determined that prolonging the vascular pedicle to include the entire femoral artery and vein, mobilized from the inguinal ligament to the knee greatly facilitated knee VCA transplantation, permitting end-to-side arterial and end-to-end venous anastomoses to the femoral vessels in the recipient hind limb.

Feasibility study

Three recipient outbred female farm pigs were used in the feasibility study. Three outbred male farm pigs were used as donors to enable lineage studies. Donor and recipient were otherwise matched in size, age, and blood type. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) approval.

Knee VCA harvest

On the day of surgery, the male donor was anesthetized with Tiletamine HCL + Zolazepam HCL (Telazol, Zoetis Inc, Kalamazoo, MI, 5 mg/kg IM), Xylazine (Xylamed, Bimeda-MTC, Cambridge ON, Canada, 2 mg/kg IM) and euthanized with Pentobarbital Sodium (Vortech Dearborn MI, 0.22 ml/kg IV). The right limb of the donor was prepped and draped in a sterile fashion. The complete hind limb was then covered with loban 2 Antimicrobial Incise drapes (3M Health Care products, St. Paul, MN). One large anteromedial longitudinal skin incision over the knee was made, curved medially proximal toward the femoral vessels distal to the inguinal ligament. The superficial femoral artery and vein were identified in the interval between the gracilis and vastus medialis muscles. They were mobilized to serve as the transplant vascular pedicle, ligating all small muscular branches

with vascular clips (LigaClips:Ethicon LLC, San Lorenzo, Puerto Rico). The femoral vessels were dissected distally until the branches of the superior genicular arteries occurred.

The anterior compartment musculature was reflected laterally from the tibia tubercle, to identify the interosseous membrane and cranial tibial artery. The nutrient foramen and associated cranial tibial vessel branches supplying bone were identified on its posterior surface and protected, as was the patellar ligament insertion on the tibial tubercle. The cranial tibial artery and vein were ligated distal to the tibial blood supply, and the tibia and fibula divided at this level, immediately distal to the tibia tubercle and nutrient foramen, at a point 6 cm distal to the joint line. The femur was easily identified from a lateral approach by dissection through the intra-muscular plane of the rectus and vastus lateralis. The femoral osteotomy was made 10cm above the joint line. All proximal muscles were released, leaving a small segment of tendon with knee transplant for later reconstruction. The dissection was completed by identification and preservation of all vascular branches of the superficial femoral artery and vein supplying the distal femur, knee joint capsule and tibia. these included the inferior and superior genicular vessels, popliteal artery and vein, and the cranial tibial vessels.

The harvested knee joint included distal femur (Fig. 1.5.), proximal tibia (Fig. 1.4), proximal fibula (Fig. 1.3), femoral artery and vein (Fig. 1.1), popliteal artery (Fig. 1.2), patella with intact cartilage and capsule The initial bone cuts were adjusted on the back table after the defect was created in the recipient for a custom fit. The tibia and femur had an intended 3cm length measured from the joint line and the fibula was shortened just below the fibular head.

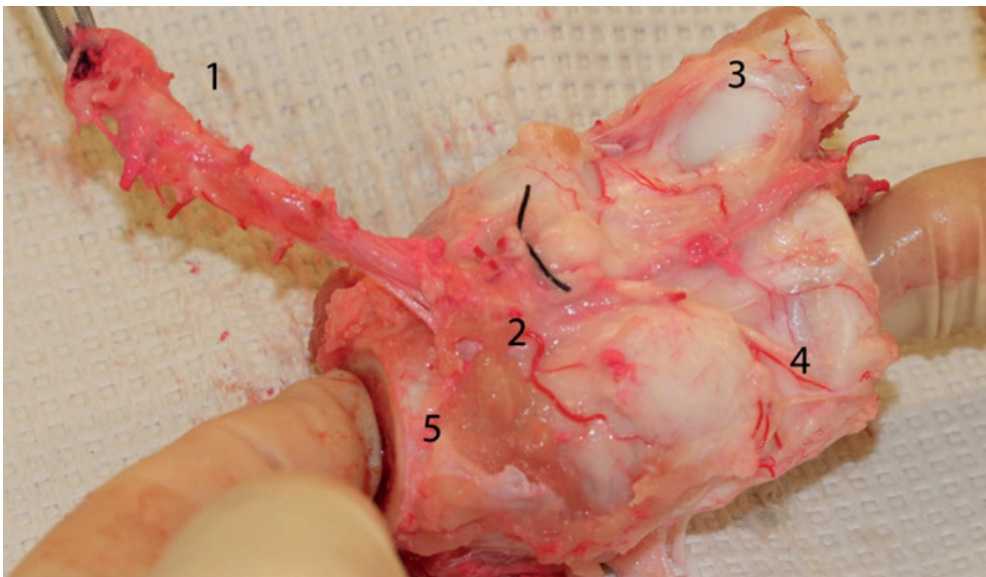


Figure 1: posterior view of whole knee joint harvested from a porcine cadaver showing: 1) allogenic vascular pedicle, femoral artery with ligated side branches 2) transition femoral to popliteal artery, with superior genicular arteries 3) fibular head 4) tibia plateau 5) femur.

Orthotopic allotransplantation

On the same operative day, a female recipient was anesthetized with Tiletamine HCL + Zolazepam HCl (IM), then intubated and maintained with inhalation anesthesia (Isoflurane 1-3%). Prior to the incision, the right leg was prepped, draped in a sterile fashion and covered with loban 2 Antimicrobial Incise drapes. One gram of cefazolin (Hospira, Lake Forest, IL) was administered intravenously for infection prophylaxis. Tacrolimus and mycophenolate were administered by a gastric tube and methylprednisone, intravenously at this time. During the entire procedure, the animal was monitored for heart rate, oxygen saturation, blood pressure, and temperature. Blood and fluid losses were compensated with intravenous administration of Ringer's solution (Baxter Healthcare Corporation, Deerfield, IL). An anteromedial skin incision over the knee was made extending medially to inguinal ligament. The femoral vessels were dissected and prepared for microsurgical anastomosis I (Fig. 2). A pedicled gracilis flap was raised by identifying the main nutrient artery (a branch of the medial circumflex femoral artery), detachment of its origin at the pelvis, and rotated around the longitudinal axis of saphenous artery and vein (Fig.3 A). The saphenous artery gives several small branches to the Gracilis flap which were left untouched (Fig.3B). The anterior compartment musculature was retracted laterally, and the cranial tibial artery ligated distally and mobilized proximally from the surface of the interosseous membrane for later implantation as an autogenous AV-bundle into the tibia (Fig. 3C). The peroneal nerve was identified and preserved. All muscle attachments to the recipient's autogenic knee were dissected directly from the bone, ensuring tendon preservation for later reconstruction. A biceps femoris muscular branch, was also mobilized for later implantation as autogenous AV-bundle into the femur. The femoral bone cut was made 3cm above the joint line, and the tibia and fibula divided at a level immediately distal to the tibial tubercle. After the bone cuts were made, the femoral artery, popliteal artery, and peroneal nerve were carefully dissected off the posterior knee to maintain limb perfusion. At this point, the knee joint was removed.

Transplantation of the allogenic knee was then performed by achieving rigid internal fixation with two retrograde placed intramedullary locked nails (I-Loc IM fixator System, BioMedtrix, Whippany, NJ). The femoral nail was placed in a retrograde fashion, introduced through a capsulotomy in the allotransplant. It was a standard 4-hole 6 X122mm nail, (Cat. No. 34-06-122). The tibia was stabilized with an antegrade-placed locked nail that was custom-made for the porcine tibia with 3 locking holes (6x122mm, Cat No. 34-06-122S). The fibula was not fixed. Intra-operative radiographs verified appropriate rod and locking pin placement. The host-transplant bone contact sites were augmented with bone graft obtained from the recipient's resected bone. Next, end-to-side arterial and end-to-side venous anastomoses were made to the superficial femoral vessels with 8.0 nylon microsuture (Ethicon LLC, San Lorenzo, Puerto Rico) (Fig. 2). All muscles were repaired to their anatomic knee insertion. The joint capsule was closed with 2- 0 and 0 FiberWire sutures (Arthrex, Naples, FL). The pedicled gracilis flap was flipped over the saphenous artery and vein to cover the entire reconstruction (Fig. 3C). A layered closure, use of Dermabond Prineo (Ethicon, LLC, San Lorenzo, Puerto Rico) and wrapping the hindlimb with Tegaderm and loban (3M Health Care, St Paul, MN) was performed to minimize the risk of deep infection. A central venous catheter (Hickman catheter, Bard Access Systems, Inc., Salt Lake City, UT) was placed in the internal jugular vein to enable intravenous drug administration and blood collection during the immunosuppressive period. A schematic overview of the complete reconstruction is

given in figure 2.

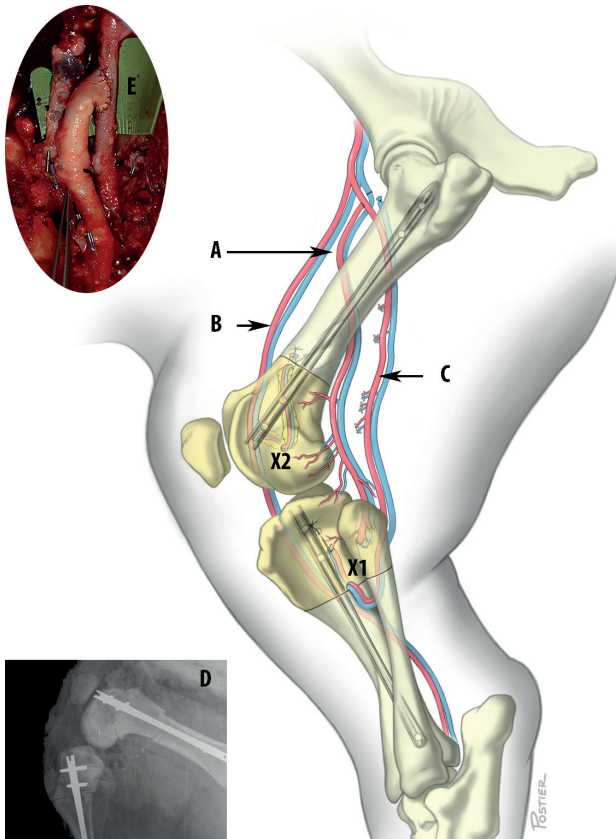


Figure 2: overview whole knee allotransplantation in hind limb model; the allotransplant included +/- 3cm of the proximal tibia, +/- 3cm of distal femur with an intact knee joint including the patella (yellow) and allogenic vascular pedicle (A). The saphenous artery left undisturbed to the lower leg (C). An end-to-side anastomosis was made from the allogenic artery to the femoral artery in the recipient (C). The venous anastomosis was made in an end-to-end fashion, both anastomoses were made using an 8.0 microsuture. The cranial tibial artery was used as an autologous arteriovenous bundle into the tibia (X1). A muscular branch of the m. biceps femorus was used as an AV-bundle into the femur (X2). The post-operative radiograph shows the final reconstruction with the intramedullary nails in place (D).

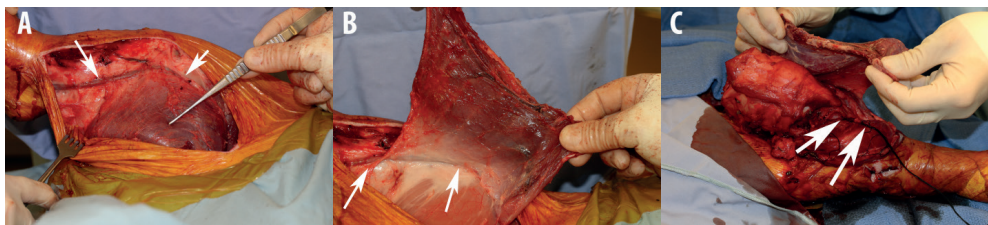


Figure 3: Plain photo-graphs taken during transplantation, showing the pedicled gracilis muscle flap in normal anatomical position from a medial view (A), raised and rotated around the saphenous artery and vein from a medial view(B), further rotated around the knee allotransplant from a lateral view (C).

Post-operative management

All animals received a 2-week immunosuppressive triple therapy consisting of Tacrolimus 0.6-1.5mg/kg (Sandoz Inc. Princeton, NJ), Mycophenolate Mofetil 30-60mg/kg (Mylan Institutional Inc., Rockford, IL) and Methylprednisolone sodium succinate (Pfizer Inc., NY, NY) 500mg start dose followed by 125mg the first post-operative day. Tacrolimus and Mycophenolate were administered orally and Methylprednisolone intravenously. Immunosuppression levels were monitored by blood draws taken every other day from a central venous catheter. Dose adjustments were made for the immunosuppression to be in therapeutic range (Tacrolimus: 5.0-15.0ng/ml, Mycophenolate 1.0-3.5 ng/ml). The methylprednisolone was tapered over the immunosuppression period (-12.5mg/day). The animals received two weeks of prophylactic antibiotic therapy with Enrofloxacin (Baytril, 7.5mg/kg, Bayer Healthcare LLC, Shawnee Mission, KS) and Ceftiofur (Excede, 100mg/kg, Pfizer Inc, New York, NY). Direct weight-bearing was allowed after recovering from anesthesia. Daily observation permitted administration of appropriate analgesics, used until no evidence of pain was observed. The animals were individually housed and received standard feed and water ad libitum. Although we had planned to briefly anesthetize all animals at 2, 4, 6, 10 weeks for radiographic views, ultrasound evaluation of the vascular pedicle, and periodic blood draws. The need to terminate the experiment due to unacceptable complications did not permit the collection of any of this data.

Sacrifice procedure

All animals were anesthetized (Telazol + Xylazine IM) and euthanized with intravenous administration of Pentobarbital Sodium (Vortech Dearborn MI, 0.22 ml/kg) as recommended by the Panel on Euthanasia of the American Veterinary Medical Association and performed according to NIH guidelines under the direction of the Institutional Animal Care and Use Committee. The femoral artery of the operated side was dissected, cannulated and flushed with heparinized saline and then injected with the contrast agent Microfil (MV-122, Flow Tech, Carver, MA). With the use of Micro-Computed Tomography (Inveon PET CT, Siemens Medical Solutions USA, Inc., Malvern, PA) at a voltage of 80 kV and 500uA with a current version of imaging software (PMOD Technologies, Zurich, Switzerland) a micro-angiography of the knee was made in a medium magnification resolution. The complete allotransplant was dissected, fixed in 10% buffered formalin for 48 hours, embedded in methyl methacrylate, sectioned in two 15um-thick sections using a diamond band saw (Exakt Technologies Inc., Oklahoma City, OK) and stained with hematoxylin/eosin (H&E).

Results

Anatomic study

The blood supply to the hind limb is provided by the superficial femoral artery and its popliteal and saphenous artery branches. The saphenous artery and vein are of great importance in our hind limb model since it provides continuous blood flow to the entire hind limb if left undisturbed. In case the femoral artery thromboses after anastomoses or manipulation, the hind limb continues to survive on its blood supply from the saphenous artery ^[16]. We were able to raise a pedicled gracilis muscle flap from its insertion and found the dominant

vascular supply to be a branch of the medial circumflex artery [17]. In addition, the gracilis is supplied by small branches arising from the femoral and saphenous arteries. Based upon the dominant vessels, we raised a wide and thin muscle flap, approximately 180mm long, 90mm wide and 10mm thick. When rotated, the flap covered the entire allogenic knee transplant. This provides both a deeper layer of transplant cover but should also stimulate some autologous angiogenesis on the surface of the allotransplant.

Surgical outcome

Three female recipients were operated at a mean age of 11 weeks with a mean weight of 26.6kg. All animals recovered extremely well after approximately 10 hours of surgery. They were able to ambulate on three feet for the first postoperative days and able to tiptoe on their operated leg. Immunosuppression levels were in the therapeutic range for the first two weeks (mean tacrolimus: 8.36 ng/mL, mycophenolate: 1.23 mcg/mL). In the first 4 days after surgery all animals developed progressively severe seromas until the point that they were not able to ambulate, but no animals were clinically showing signs of infection or rejection at that moment and remained good appetite. Daily observation, monitoring, and administration of antibiotics and analgesics were performed in close collaboration with comparative medicine. Over time, the seromas did not resolve spontaneously, as we have observed in our previous tibia defect models. The severity of the seromas and inability to walk resulted in pressure wounds on the medial malleolus and subsequent infection of the seromata. In collaboration with comparative medicine and in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines, the animals were sacrificed at the end of the second week after transplantation according to the sacrifice protocol.

Evaluation of available data

All vascular pedicles were patent at the time of sacrifice, confirmed with microangiography (Fig. 4) and demonstration of contrast agent in the tissue seen both on gross visual inspection and in histologic slides (Fig.5.1). Histologic evaluation of the allogenic vessels showed no signs of intima hyperplasia, frequently observed in more chronic transplant rejection (Fig. 5.1). Osteocyte, cartilage, and epiphyseal viability was maintained (Fig. 6). However, widespread infiltration of leukocytes and lymphocytes was found in all specimens (Fig. 7). Differential leucocyte counts over the brief survival period revealed an increase in white blood cells and higher percentages of neutrophils 2 weeks following transplantation, and at time of sacrifice due to the infected seromas (Fig. 7). Prior to surgery the median passive knee range of motion was 40 degrees, after two weeks 32.77 degrees, and at time of sacrifice 37.5 degrees.

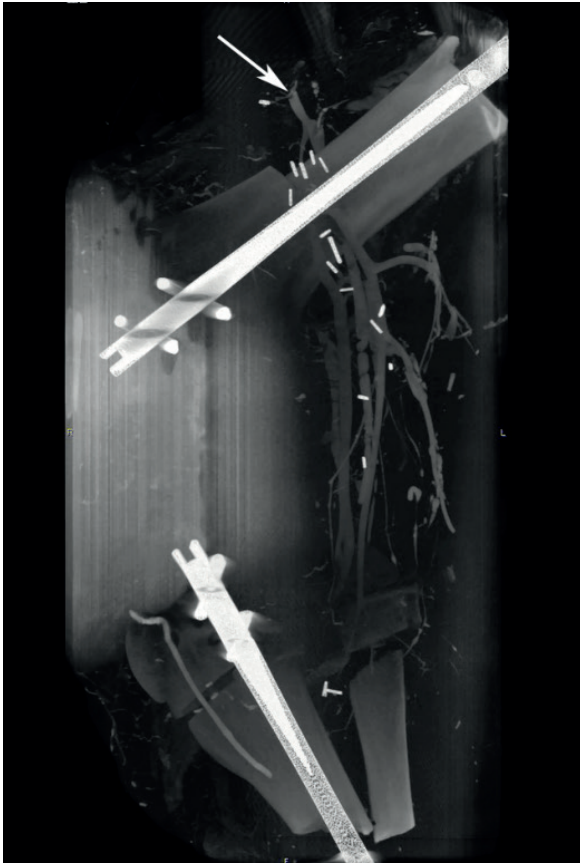


Figure 4: Micro angiography of the allotransplant, the contrast agent inserted in the femoral artery (white arrow) showing the allogenic pedicle was patent at time of sacrifice.

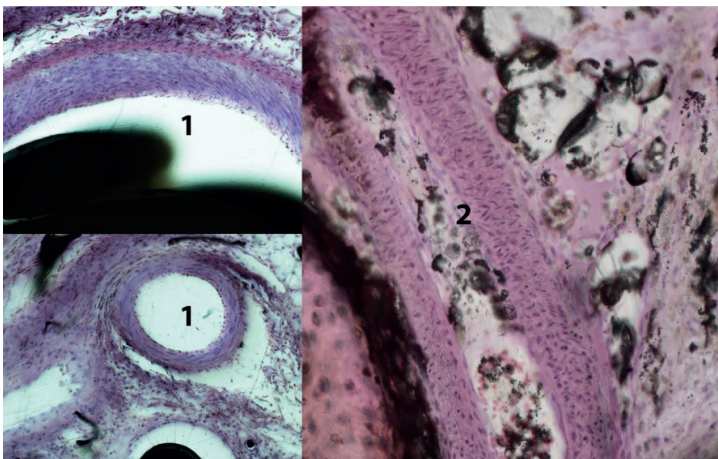


Figure 5: Hematoxylin and eosin stain (10X) vessels within the allotransplant (1), compared to normal vascular structures in the porcine knee (2), no intima hypertrophy was observed at time of sacrifice.

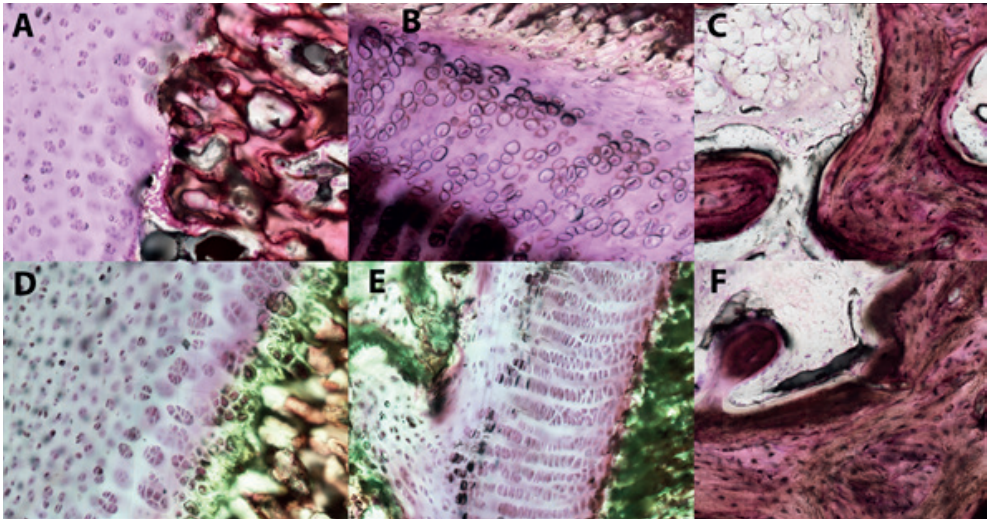


Figure 6: Hematoxylin and eosin stain (10X) of allotransplant cartilage (A), epiphysis (B), bone and bone marrow (C) compared to normal cartilage (D), epiphysis (E), bone and bone marrow (F), viability of these tissue types seems to be maintained at time of sacrifice.

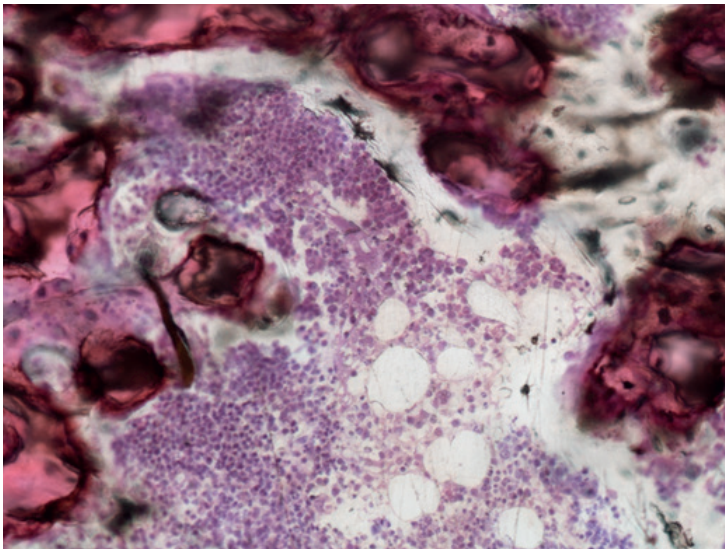


Figure 7: Hematoxylin and eosin stain (10X) of allotransplant bone and bone marrow, showing infectious changes by leukocyte and lymphocyte influx.

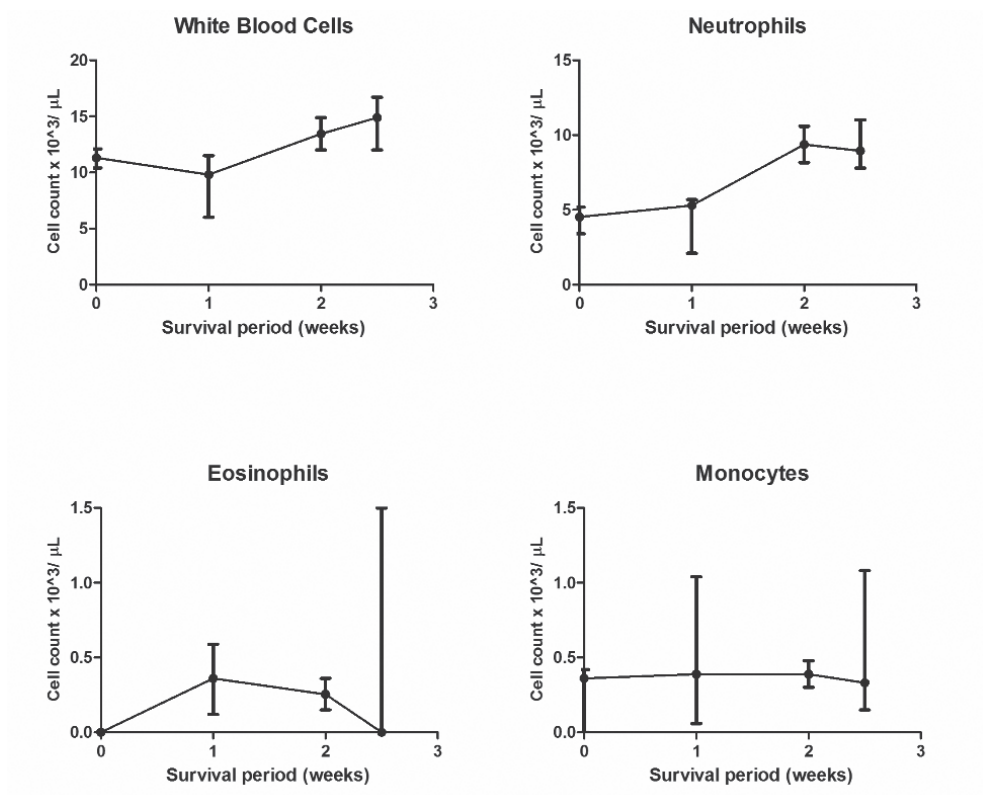


Figure 8: blood cell counts at base line, 1 and two weeks after transplantation followed by time of sacrifice (last time point).

Discussion

Allotransplantation of vascularized bone, joint, face and hands present an ethical dilemma [14, 18]. The risks, expense and complications of life-long immunosuppression need to be carefully considered in these non-life-critical transplantations, particularly if other reconstructive methods are possible. Only a few allogenic knee joint transplantations have been performed in human. All of these clinical attempts have had poor outcomes, ultimately requiring an above-knee amputation [1, 5, 7, 8, 10]. Given the clinical outcomes reported to date, joint allotransplantation at present remains an unproven reconstructive method.

Experimental whole knee joint transplantation has been performed in several large and small animal models, showing the procedure to be technically feasible [12, 15, 19, 20]. In a canine knee joint model, vascularized autografts healed and functioned without problem, but identical allotransplants were rejected within a few days in the absence of immunotherapy [12]. Vascular endothelium is highly immunogenic, causing vascular thrombosis and death of the allotransplanted tissue days to a few weeks without adequate immune suppression [1, 10, 12].

In order to avoid the risks life-long drug therapy, we have developed a novel method to maintain bone and joint allotransplant viability by rapid development of an autologous neoangiogenic circulation [21-24] [25] [26-29]. We tested this novel method in small animal models with bone only VCAs. At the time of the transplant procedure, implantation of autogenous AV-bundles within bone results in capillary sprouting and ultimately, a new autologous neoangiogenic circulation within the allotransplant. Viability is maintained in the short-term by 2-weeks' triple drug therapy. These drugs not only prevent acute rejection but also promote neo-angiogenesis from the implanted AV bundle [14, 26, 30]. Bone blood flow is maintained despite eventual nutrient vessel thrombosis, and new bone forms to heal and remodel the transplant. Osteocytes within the new bone have been found to be autogenous to the recipient animal.

Similarly, whole knee joint VCA allotransplants have been studied in rat and rabbit models. Although viability was maintained, the knee allotransplants developed both arthritic changes and joint contractures^[14]. The cause of the degenerative changes requires further investigation. The potentially affected by loss of proprioceptive nerve supply, as well as poor cartilage nutrition caused by a relatively fixed flexion deformity in these small animals. Joint denervation certainly could lead to joint destruction, termed Charcot arthropathy. These findings are similar to those found in clinical experience with long-term immunosuppression [1, 7]. Future research should also focus on the role of joint denervation and subsequent development of degenerative changes.

In this article, we report a new whole knee VCA model to permit future investigation of best methods used to reconstruct large bone and joint defects in a large animal model. The pig is certainly one of the best experimental VCA models for translational studies of methods showing promise in small animal models [16]. Adult pigs are of similar size to man and have bone and joints of similar anatomy. Additionally, the porcine blood type, and swine leukocyte antigens (SLA) are well known [16, 31]. This allows evaluation of transplantation in size, blood type and age matched animals but over a major mismatch in histocompatibility.

The development of a larger species whole knee joint allotransplantation allows pre-clinical evaluation of functional, biomechanical, histological, immunological, radio graphical, and neo-angiogenesis analyses of joint VCAs, all in one animal. Bone healing, remodeling and neo-angiogenesis can be confirmed with the use of serial radiographs, micro-CT and quantitative histomorphometry. Biomechanical data can be obtained in measuring the range of motion of the knee during the survival period. At the final evaluation axial-compression, cyclic reference point indentation and torque force testing can be performed in the same specimen. Serial blood draws allow monitoring of blood cytology and systemic immune response over time. Local immune response and gene-expression can be evaluated by immunohistochemistry and real-time PCR. With the use of laser capture microdissection and qPCR, the cell population and re-population of the allotransplant can be determined in specific cell lineages of interest by quantification of sex-determine region of the Y-chromosome (*SRY*), if a sex-mismatched set-up is used.

As there were no problems or complications during surgery or in the early post-operative period (0-4 days), we believe the procedure to be technically feasible although clearly complex, requiring experienced surgical teams. The post-operative care of the animals is extremely important, as we have previously experienced in our tibia defect models [16, 25, 32]. Immediate post-operative ambulation was observed following tibia allotransplantation, including full weight bearing after an average of only 4 days. In this feasibility study, the animals were also standing and walking within the first few post-operative days without limitation. Wound problems, including the development of large seromas with infection developed, requiring termination shortly after 2 weeks. Future use of the same animal model will require some modification to reach the target survival period. Wound drainage with both deep and superficial post-operative drains may prevent seroma development, and protection from full weight-bearing, implant failure. Thereafter, some form of protection will be necessary until tissue stability has been achieved. This may include the use of an abdominal sling to suspend the hind limb. The observed complications were unexpected, and we believe avoidable in the future, based upon our prior experience of hind-limb bone allotransplantation in the same porcine model.

The pedicled gracilis muscle flap in porcine, has been described in several porcine studies [17, 33]. In our anatomy and feasibility study, we found the same vascularization pattern as described in the literature. We used the anatomy to elevate a pedicled gracilis flap for soft tissue coverage and induction of autologous revascularization of the allogenic knee. In previous studies, bone viability was maintained solely by autogenous AV-bundles. Although this might be true for bone only VCA's, cartilage is dependent on diffusion as it does not contain its own blood flow, and the knee capsule is dependent on the allogenic microvasculature. The autogenous AV-bundle implantation might therefore not be enough to maintain viability thought-out all tissue types. The gracilis muscle flap was used to stimulate and induce formation of an autogenous neoagenic circulation in all tissue types without the need for life-long immunosuppression. In this, study the allotransplants appeared viable two weeks after transplantation. In future research, a detailed micro angiography of the complete hind-limb should be made to assess neo-angiogenesis arising from the AV-bundles and muscle flap into multiple tissue types. Thereafter, viability can be assessed through histology and molecular analyses.

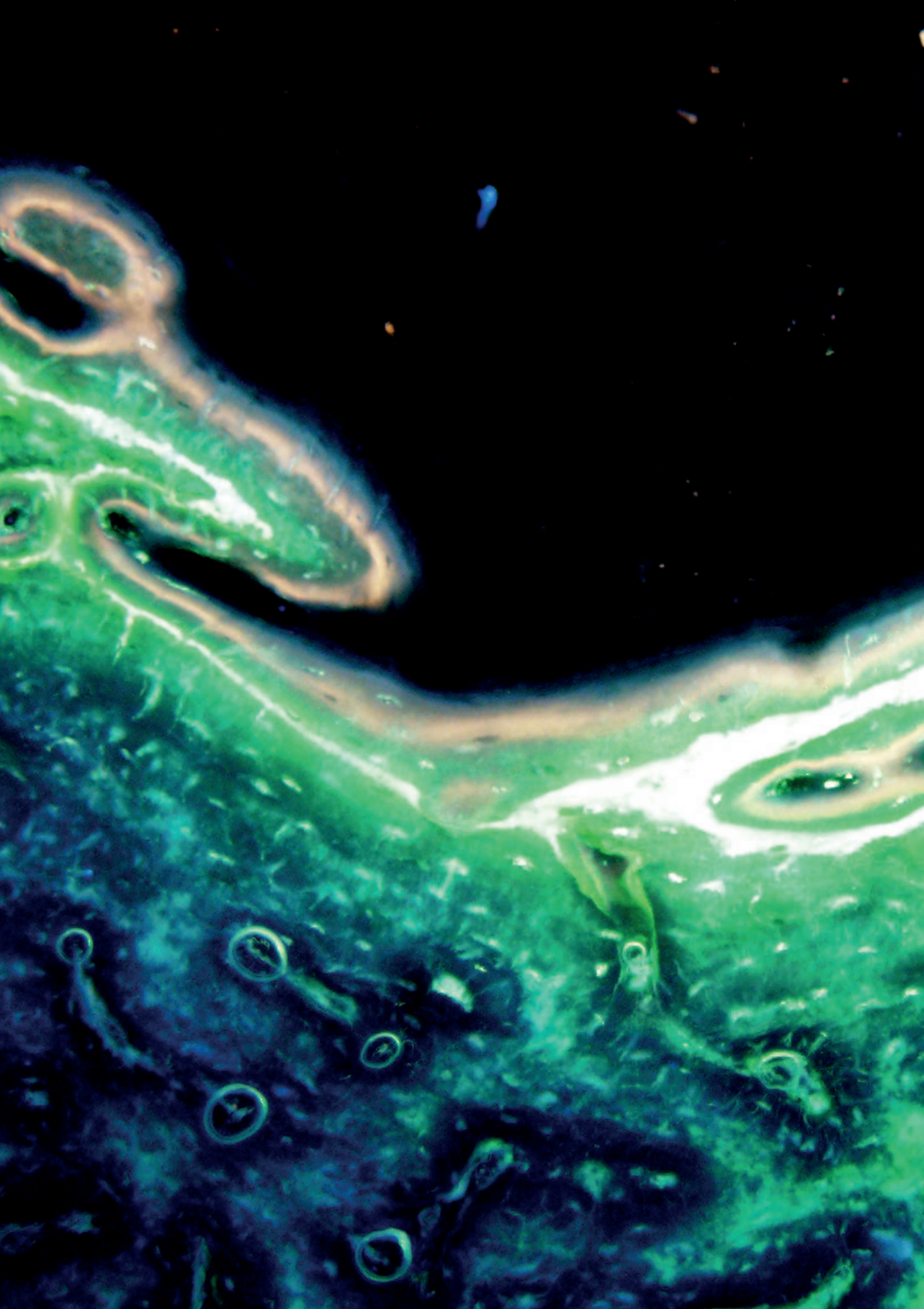
We propose a new whole knee joint allotransplantation model with surgical induced neo-angiogenesis and short-term immunosuppression. Our current surgical technique works, whereas the post-operative care and treatment needs improvement in future studies. This model would provide critical pre-clinical information and allows multiple analyses of the whole knee allotransplant after cessation of immunosuppression and evaluate surgical induced autologous revascularization.

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CHAPTER 8

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9

CHAPTER

Summary and
general discussion

The overarching aim of this thesis was to microsurgically transplant living allogenic bone and joint while maintaining viability without the need for life-long immunosuppression in a pre-clinical animal model. In this chapter, the main findings of the studies are summarized, discussed and suggestions for future research are given.

Summary and conclusions

Part I:

The first part of this thesis focuses on the current clinical outcomes of autogenous vascularized bone grafts (VBG) for the reconstruction of large segmental bone defects in the lower extremity. We focus on the outcomes of VBGs used alone or in combination with a massive allograft. The eventual goal is limb-salvage and preservation of form and function, while minimizing complications and re-operations. We hypothesized that segmental bone defects are best reconstructed with a vascularized autograft combined with or without a massive cortical allograft.

In **Chapter 2**, we performed a review of literature on the outcomes of VBGs for the reconstruction of large segmental defects in the lower extremity. Segmental bone loss is often the result of trauma, primary malignant tumor resection, meta-static tumor resection, infection, failed primary reconstruction, or congenital pseudarthrosis. Treatment of these large bone defects is challenging. Historically, vascularized bone grafts have been used since the 19th century. The desirability of a vascularized bone graft above a non-vascularized bone graft has been emphasized by several early researchers ^[1-3]. Due to the rapid development of microsurgical techniques the free fibula flap has become one of the most important VBG for the reconstruction of segmental bone defects. The first successful free vascularized fibula transfer at Mayo Clinic was performed in 1979 by Dr. M.B. Wood. The structure and shape of the fibula make it useful for diaphyseal reconstruction; a straight segment of bone between 26 and 30 cm can be harvested, and stability can be obtained with rigid internal fixation to the recipient bone. Vascularized bone flaps remain viable due to microvascular repair of the nutrient pedicle of the bone, permitting the survival of osteogenic cells ^[4, 5]. The viability of VBG results in faster union rates, fewer fatigue fractures, rapid remodeling and hypertrophy, and less resorption compared to non-vascularized bone transfer ^[6-8]. Primary union rates of 67-84% have been found in the literature, however after secondary bone grafting, the rate of union is 81-92% ^[9-11]. Complication rates after vascularized bone grafting for reconstruction of a bone defect in the lower extremity are strongly dependent on the location and underlying pathology of the defect. Risk factors for poor outcomes are post-operative chemotherapy, tobacco use, and a history of osteomyelitis ^[9, 11].

Free fibula flaps often poorly match the diameter of the defect in the lower extremity. Therefore, they are susceptible to early stress fractures in the first year due to insufficient protection from mechanical overload. Combining vascularized fibular grafts with a massive allograft shell can reduce the mechanical load. This technique has been known in literature as the Capanna technique ^[12]. In **Chapter 3**, we performed a systematic review of literature on the outcome of this technique. We found that combining a massive allograft and an intramedullary vascularized

fibula as a method for the primary reconstruction of segmental bone defects in the lower extremity provides a single step reconstruction with good functional long-term outcomes. This technique has a primary union rate of 86.5%, with a high re-operation and complication rate (Chapter 3). This technique should especially be considered in tumor cases, since there is little evidence in literature for its use in traumatic or congenital defect cases.

In conclusion, the use of autogenous vascularized bone grafts remains the gold standard for large segmental bone defects in the lower extremity with or without the use of a protective massive allograft shell. Primary union rates are significantly higher compared to non-vascularized massive allografts alone. Vascularized bone grafts are limited by the few expendable donor sites and donor site complications.

Part II:

In the second part of the thesis we focused on a new experimental reconstructive method: living bone allotransplantation, which is a form of vascularized composite allotransplantation (VCA). While VCAs are currently limited by the use of life-long immunosuppression, we developed a new experimental method to maintain bone VCA viability. This novel method of living bone allotransplantation combines the microvascular repair of the nutrient circulation with implantation of recipient-derived arteriovenous (AV) bundles with short-term immune modulation. We hypothesized that bone VCA viability can be maintained in a large animal model using surgical angiogenesis and short-term immunosuppression without tolerance induction or other permanent immune modulation. Instead, new bone formation in transplanted allogeneic bone is the result of transplant chimerism.

In **Chapter 4**, we demonstrated the ability to orthotopically transplant bone-only VCAs across a major histocompatibility barrier using sex-mismatched Yucatan miniature swine, implanting an AV-bundle within the medullary canal to induce recipient-derived angiogenesis (surgical angiogenesis). Two weeks of immunomodulation were used. We implanted a patent AV-bundle in group 1 and a ligated AV bundle in group 2 as a no-angiogenesis control. In both groups, a microsurgical repair of the nutrient vessel was performed in the recipient, combined with rigid internal fixation of the allotransplant. We successfully performed 12 vascularized bone allotransplantations. Over a 20-week period, pigs gained an average of 20 kg (40 kg final weight) and full-weight bearing was observed without exception by four days after operation. In this study, we found new periosteal bone formation and subsequent bone healing to result from blood flow through the microsurgically repaired allogenic vascular pedicle, confirmed by allogenic pedicle patency in the first 4-6 weeks after transplantation. The implantation of an autogenous AV bundle has no adverse effect on material properties but has a positive effect on bone remodeling of endosteal surfaces despite thrombosis of the allogenic pedicle.

CHAPTER 9

The extent of surgical neo-angiogenesis and the effect of it on transplant viability and gene expression was evaluated in **Chapter 5**. In this chapter, we found significantly greater amounts of medullary vessels in the patent AV bundle group 20-weeks after transplantation. Endosteal osteocyte counts were highest in this group. A significant increase in the expression of endothelial growth factor like-6 (*EGFL-6*) was observed, with a positive correlation with measured vessel volumes.

The sex-mismatched nature of our large animal model allowed for evaluating the extent of transplant chimerism by investigation of the *SRY* gene with RT-qPCR. Additionally, further development of Laser Capture Microdissection techniques combined with fluoroscopy allowed precise sampling of osteocytes. In **Chapter 6**, we studied cell lineage within sex-mismatched bone VCAs by LCM (Laser Capture Microdissection) and RT-qPCR. Analyses of areas of new bone formation showed significant levels of microchimerism, demonstrating new bone formation to result from the migration of autogenous cells. Some allogenic male donor cells survived, evidenced by RNA analyses. No systemic chimerism was found in liver and spleen, indicating that induction of donor specific tolerance was not important.

Only two weeks of immunosuppressive triple therapy was used in our large animal model. After cessation of the immunosuppression, recipient rejection of the VCA would be expected. In **Chapter 7**, we measured the systemic immune response and effect of AV bundle implantation on local immune status of the allotransplant 20 weeks after transplantation. Thrombosis of the allogenic vascular pedicle occurred 4-6 weeks after transplantation, as found in Chapter 4. Histologic evaluation of the allogenic pedicle confirmed loss of pedicle patency due to intimal hypertrophy, as expected without immune modulation. Necrosis of the bone does not occur due to the development of a neo-angiogenic autogenous blood supply during the initial period of drug therapy. In this study, there was no adverse systemic effect of this process, studied by periodic peripheral blood cytokine determinations during a 20-week survival period. The allotransplants themselves demonstrated less inflammation, less necrosis and improved viability with the addition of an autogenous AV bundle. All implanted patent AV bundles in were patent 20 weeks after transplantation and showed significant spouting into the allotransplant as demonstrated in Chapter 5. Histologic evaluation of the AV bundle showed a normal patent arterial wall.

In conclusion, this novel approach to bone allotransplantation seems to have significant advantages over conventional VCA methods proven in a pre-clinical experimental animal model. The data found throughout chapters 4-7 are consistent with those found in previous studies using the same novel method to maintain transplant viability in small animal bone-only VCA [13-19]. The porcine model has distinct advantages for allogenic tissue transplantation research in order to retrieve pre-clinical translatable results. Their size, anatomy, physiology and immunology are well known and comparable to human. Our porcine tibia defect model has proven to be a great asset for VCA research and produces consistent and reproducible results [20-22]. Living bone allotransplantation with short-term immunosuppression and AV bundle implantation holds therefore clinical potential using our novel method.

Part III:

In the third part of this thesis, we extrapolated our novel method of surgical neo-angiogenesis to a novel large animal model to study orthotopic vascularized whole knee joint allotransplantation. We hypothesized that surgical angiogenesis will preserve viability, permit healing and maintain articular function of whole knee joint allotransplantation with short-term immunosuppression.

In **Chapter 8**, we performed an anatomy and feasibility study on allotransplantation of a vascularized whole knee joint allotransplantation with autologous AV bundle implantation covered by a pedicled gracilis muscle flap in three outbred farm pigs. The complexity, expense and postoperative complications resulted in premature termination of the experiment. We conclude whole joint allotransplantation is an extremely complex surgical procedure, wherein our new model requires further development to establish a valid large animal model with reproducible results.

Discussion

Revascularization of conventional non-vascularized bone allografts, often cryopreserved (CBA), is a slow and incomplete process. Almost 40-50% of the interstitial lamellae remain avascular after two years ^[23-25]. In the past decades, autogenous vascularized bone grafts have been widely used. The advantage of this technique is better and faster incorporation of the graft ^[10, 26-30]. The free fibula flap has become the workhorse in clinical setting for the treatment of large segmental bone defects. However, the initial strength of the vascularized fibular graft may be insufficient in lower extremity reconstructions, due to small diameter of the bone. Although, hypertrophy often occurs at an average of 18 months after surgery, hypertrophic bone fractures have been reported ^[7, 31]. A vascularized fibula graft in the lower extremity should be protected against fatigue fracture in the first few years ^[32]. To solve these problems, Dr. Capanna introduced a new technique wherein the vascularized fibula graft is combined with a massive allograft to protect the fibula from mechanical loads (VBG + CBA) ^[12]. Alternatively, living bone and joint allotransplantation offers a novel method to reconstruct large segmental bone defects. Here we will discuss the different treatment modalities of using vascularized autogenous bone and vascularized composite allotransplantation of bone and joint.

Mechanical stimulation or stress loading of bone is a major factor in the maintenance of normal balance between bone formation and resorption. This process can take place at different levels of the bone segment according to the individual characteristics and variation over time of the axial strain rate ^[7, 33, 34]. Increased mechanical load on long bones can produce a stimulus in which bone formation outpaces bone resorption ^[35]. Correspondingly, repeated mechanical load which exceeds the strength of the bone can cause stress fractures. Bone growth is evidenced in the areas where compression or traction load is increased, meanwhile decreasing load leads to resorption ^[34]. The ability of a vascularized or non-vascularized bone to resist and adapt to mechanical loading depends upon both its strength and viability ^[4, 36].

Vascularized fibula flaps used for the reconstruction of a large segmental defects alone demonstrate hypertrophy as a response to stress loading in 80% of cases at 24 months after surgery [7]. When a VBG is combined with an CBA, the VBG substantially improves the biological properties of the reconstruction due to the independent vascularity. In short-term, the VBG induces fusion and incorporation into the recipient bone. In long-term, the VBG interacts with the allograft by inducing revascularization of endosteal surface of the CBA [34]. The endosteal surface of the CBA otherwise remains avascular and therefore represents an element of lesser mechanical resistance [24, 37].

Computed tomography studies of a combined VBG and CBA reconstruction demonstrate this effect in three different remodeling patterns depending upon the variation of the load on the reconstruction. If the CBA remains intact, the mechanical stress on the vascularized fibula remains mild and constant. In response, the VBG will show an increase in diameter without cortical thickening (pattern 1). Osteo-inductive processes have been found in areas where the growing vascularized bone comes into contact with the endosteal surface of the CBA. The second pattern is associated with a stress fracture of the CBA. Due to the suddenly increased axial strain on the VBG, the plastic properties of the hypertrophying VBG becomes heightened. As a result, an increase in diameter is observed together with significant cortical thickening. The osteo-inductive processes found in these cases are more intensely activated at the CBA level. This leads to a complex process of osteogenic substitution [34], but hypertrophy of the VBG together with revascularization of the CBA is preferable. It has been described in the literature that in some cases of combined VBG and CBA reconstruction, the complete CBA shell had been reabsorbed. The reabsorbing process had occurred simultaneously with increase hypertrophy of the VBG [34].

The biological behavior of the VCA with short-term immunosuppression and surgically-induced neo-angiogenesis was compared to the biological behavior of VBGs, CBAs or a combination of VBG and CBA. We found in our experimental VCA study that no fractures occurred during the 20-week survival period and all proximal host-transplant interfaces demonstrated complete union. In our porcine tibia VCA defect model, we used rigid internal fixation with dual locked LCP plates. Only one uni-cortical locking screw was used to hold the allotransplant in place. Inability to limit activity in our pig hind-limb model is a potential explanation for the three cases of incomplete distal union. We observed periosteal new bone arising from the VCA sufficient to form a bridging callous. This healing process is comparable to the healing process of autogenous VBGs [7]. Change of mechanical bone properties have been reported after both VBG and CBA reconstruction [38] that. Bone material properties within the allotransplants changed 20 weeks after transplantation compared to normal (contra-lateral) bone while bone mineral density (BMD) was maintained. Mechanical protection of the allotransplants by the internal fixation and limited follow-up are possible explanations for the changed mechanical properties.

Implantation of a VBG within a CBA has been reported to increase the biologic properties of the reconstruction and improve outcomes [12, 34]. Biologically, the revascularization of the CBA by a VBG can be compared to autogenous revascularization of a VCA with AV bundle implantation, wherein the VBG acts as the AV bundle. Living bone allotransplantation provides immediate bulk

and stability, while maintaining the biological properties of VBGs. The distinct difference between the two methods is that VCAs do not require a donor site.

Allotransplantation of complete knee joints is a novel approach to knee replacement surgery. Vascularized composite allotransplantation of bone and joint has been performed clinically [39, 40]. In these case series, multi-organ donors were used after explantation of vital organs. After harvest, the knee joint VCA was adjusted to fit the defect of the recipient on a back table. After transplantation and anastomosis of the vascular pedicle, internal fixation was performed by retrograde placed intramedullary nails. After initial success, all knee joint VCA attempts failed. In four patients, the VCA was rejected and had to be partially removed. Subsequently, a total knee arthroplasty was performed which led to an infection and eventually amputation [40]. Long-term immunosuppression and loss of transplant viability due to rejection should be alternatively managed to make allotransplantation of whole knee joints a success.

Whole knee joint allotransplantation using our novel method of maintaining VCA viability by surgical neo-angiogenesis without the use of life-long immunosuppression has been evaluated in a rabbit model. Surgical neo-angiogenesis resulted in improved viability, bone remodeling, and bone properties. Flexion contractures, due to in part to the hyperflexed resting stance of the rabbit knee, caused functional impairment [41, 42]. The desirability to perform this type of VCA research in a large animal would facilitate thorough investigation of whole knee joint VCA feasibility, including bone healing, joint properties, tissue perfusion, as well as systemic and local immune responses. Although the porcine tibia defect model has proven to be of great value for bone-only VCA research, the novel large animal model for knee joint VCA research needs to be further developed. The surgical ability to successfully perform whole knee joints has been confirmed by both experimental and clinical studies [43, 44]. Post-operative management however should be adjusted and further studied.

In the ideal world the application of living bone and joint allotransplantation would be further evaluated in an experimental follow-up study. For bone-only allotransplantation, I would like to see value and issues with allotransplantation versus current treatment. I would probably do a long-term survival experiment comparing conventional reconstruction with cryopreserved bone versus bone allotransplantation in pig model using our novel method of maintaining transplant viability. Second, I would like to see value and issues with surgical angiogenesis versus usual VCA methods, compare bone allotransplants with long-term IS versus our novel method, also with long-term survival in pig model. For whole joint allotransplantation I would like to do an experimental follow-up study in which we transplant an entire knee joint and optimize the post-operative care by using drains and provide support by using an abdominal sling. When we have a working and validated joint allotransplantation model, we can compare non-viable allograft whole joint with vascularized allotransplantation whole joint in a pig model. Although this type of research would greatly facilitate further development and pre-clinical data to safely translate the results to clinical setting. There are several major limitations to this type of research, the expense related to immunosuppressive drugs and long-term survival time of large animals are a major concern. Additionally, pigs are intelligent animals who are hard to compel to take their drugs for an extended period of time.

CHAPTER 9

Alternatively, one could start experimenting on humans since living bone allotransplantation has the potential for clinical application using our novel method to maintain viability. The technical feasibility of living bone allotransplantation has now been shown in humans and multiple small and large animal models. Additionally, pre-clinical experience with VCA using our novel method is very promising with reproducible results, although found in a low number of experimental cases. Clinical experience with living bone allotransplantation has resulted in one successful transplantation to date ^[45]. In another case, the recipient of an allogenic vascularized femoral diaphysis was transfected with a cytomegalovirus ^[39]. Thus, an optimum of hygienic security has to be claimed for recipients of living allogenic bone. Therefore, transplantation protocols, careful case selection, transplantation safety and concerns with immunological hazards should be further evaluated. Prior to clinical application, several human cadaver studies should be conducted to develop transplantation procedures and study the human vascular anatomy of different bones (donor sites). First a review of literature should be performed on the current knowledge on vascular anatomy of different bones and joints. Second, cadaver dissection can be performed by injecting an upper or lower extremity with Microfil (contrast agent which hardens after injection), perform imaging studies (micro-CT), and dissect the limb thereafter. This way multiple expandable donor sites can be investigated.

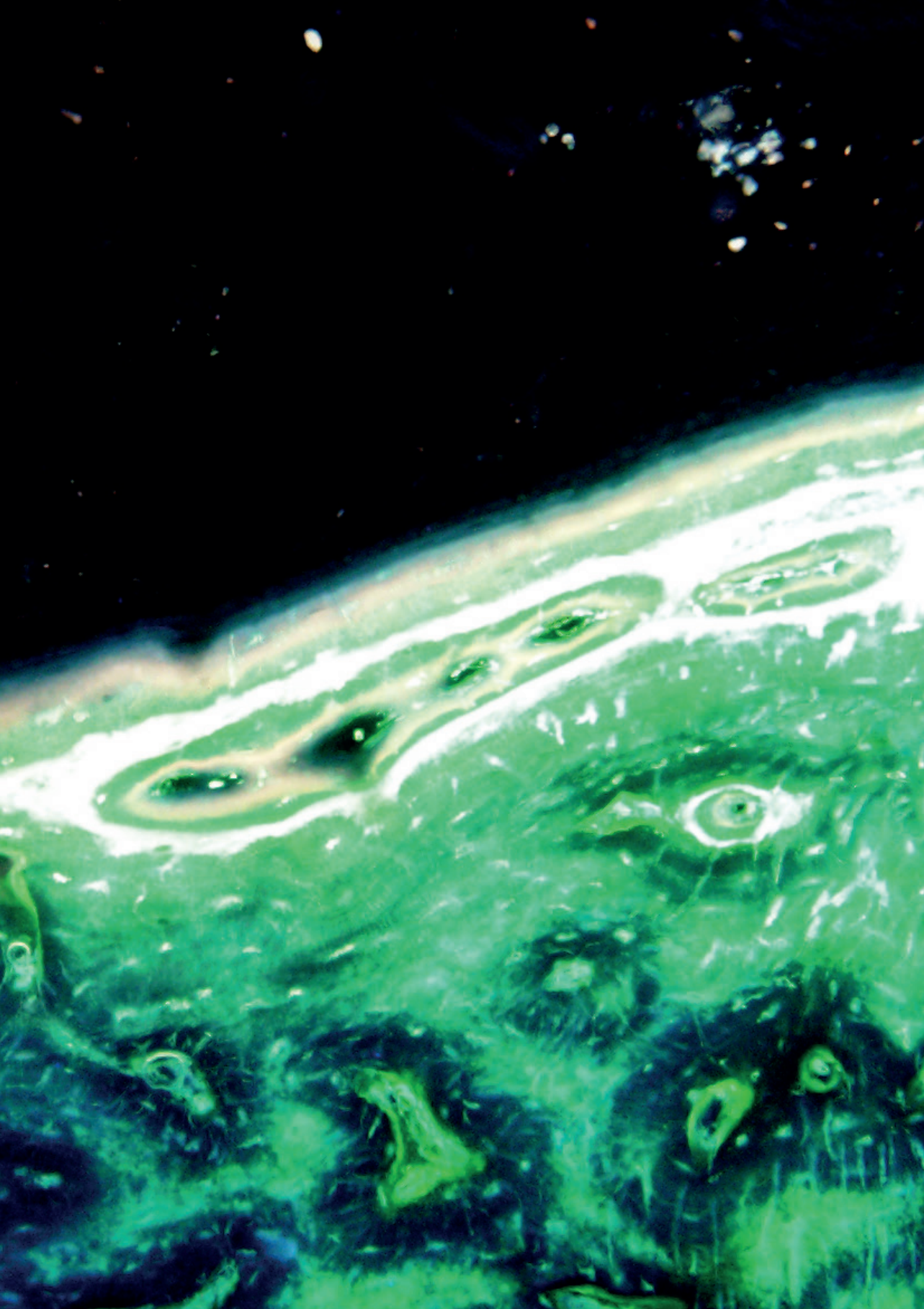
In the future, we expect vascularized composite allotransplantation to be more widely used and accepted. Clinical experience with hand, face and abdominal wall has been the forefront of VCA developments after organ transplantation. With the lessons learned from previous VCA research, and the implementation of our novel method, we provide possible new option for reconstructive surgery. It is essential to acknowledge that reconstructive surgery is not life-saving surgery, therefore the risk to the patient should be minimized. Do no further harm.

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10

CHAPTER

Nederlandse samenvatting

Het overkoepelende doel van dit proefschrift was om microchirurgisch gevasculariseerde allogene bot en gewrichtstransplantaties uit te voeren in een preklinisch diermodel met slechts kortdurende immunosuppressiva en behoud van weefsel vitaliteit door chirurgisch geïnduceerde autologe neo-angiogenese. In dit hoofdstuk wordt een samenvatting gegeven van alle hoofdstukken, en de daarbij behorende conclusies besproken.

Deel I:

In het eerste deel van dit proefschrift worden twee studies gepresenteerd over het klinische gebruik van autoloog gevasculariseerd bot voor de reconstructie van segmentale botdefecten in de onderste extremiteit. Hierbij werd de focus gelegd op het gebruik van gevasculariseerd autoloog bot (VGB) alleen of in combinatie met een niet-gevasculariseerd stuk corticaal donor bot (CBA). Het uiteindelijke doel bij reconstructie van het onderbeen is behoud van vorm en functie terwijl het risico op complicaties en re-operaties zo klein mogelijk wordt gehouden. Als hypothese stelde wij dat segmenteel bot verlies het beste reconstrueert kunnen worden met gevasculariseerd bot (VGB), of in combinatie met een niet-gevasculariseerd stuk donor bot (CBA).

In Hoofdstuk 2, beschrijven wij een review over de uitkomsten van het gebruik van VGBs voor de reconstructie van grote segmentale bot defecten in de onderste extremiteit. Hiervoor zijn voornamelijk artikelen gebruikt die binnen de Mayo Clinic zijn gepubliceerd.

Grote segmentale defecten van bot kunnen ontstaan als gevolg van trauma, resectie van bot tumoren, bot metastasen, infectie, gefaalde eerdere reconstructie pogingen, en congenitale pseudartrose. Gevasculariseerd bot wordt sinds halverwege de 19e eeuw gebruikt, echter bestaat het verlangen naar gebruik van gevasculariseerd bot boven dat van niet-gevasculariseerd bot sinds het begin van de 19e eeuw [1-3]. Door de snelle ontwikkeling van de operatiemicroscoop en microchirurgisch instrumentarium is verplaatsing van gevasculariseerd weefsel mogelijk geworden over het gehele lichaam. Op deze manier is het mogelijk om bijvoorbeeld de fibula (een van de twee onderbenen botten) aan te sluiten op een plek elders in het lichaam. Deze techniek is ook bekend als de vrije fibula lap en is een vorm van gevasculariseerd autoloog bot (VGB). Deze methode is een van de meest belangrijke reconstructie methode voor de behandeling van grote segmentale bot defecten. De eerste succesvolle vrije fibula flap in de Mayo Clinic was in 1979 door Dr. M.B. Wood. De structuur en vorm van de fibula maken dit bot geschikt voor de reconstructie van bot defecten. Er kan namelijk een recht stuk bot worden geoogst van ongeveer 25-30 cm, en stabiliteit worden verkregen door interne fixatie aan het bot waar het defect zich bevindt. De osteocyten (bot cellen) in gevasculariseerd bot blijven vitaal nadat het bot verplaatst door microchirurgisch herstel van de bloedvaten [4, 5]. De vitaliteit van VGB resulteert in snellere genezing, minder stressfracturen, snellere remodelering en hypertrofie, en minder bot resorptie vergeleken met CBAs [6-8]. In 67-84% van de gevallen waar VGBs zijn gebruikt vindt primaire bot genezing plaats. Secundaire bot genezing vindt plaats na re-interventie in 81-92% van gevallen [9-11]. Complicaties geassocieerd na VGB voor de reconstructie van een groot bot defect in de onderste extremiteit is sterk afhankelijk van de locatie en onderliggende pathologie van het defect. Risicofactoren die geassocieerd zijn met slechtere uitkomsten zijn het gebruik van postoperatieve chemotherapie, roken, en patiënten die een defect hadden door een infectie [9, 11].

De vrije fibula flap is qua vorm en grootte vaak een minder goede match met het defect in de onderste extremiteit. Hierdoor is de reconstructie met een vrije fibula vatbaar voor stressfracturen in het eerste jaar na operatie als het niet goed beschermd wordt tegen mechanische belasting. VBGs kunnen worden beschermd tegen grote mechanische belasting door ze te combineren met een groot stuk niet gevasculariseerd bot (CBA). Deze techniek van VBG en CBA in een operatie is ontwikkeld door Dr. Capanna.

In Hoofdstuk 3, hebben we een systematische review van de literatuur verricht die gaat over de uitkomsten van de gecombineerde VGB en CBA-reconstructie van grote bot defecten in de onderste extremiteit. Uit deze studie bleek dat de combinatie van VBG en CBA lijden tot een goede primaire reconstructie, met goede functionele uitkomsten op lange termijn. Deze techniek leidt tot primaire botgenezing in 86.5% van de gevallen. Echter is deze techniek geassocieerd met een hoge re-operatie en complicatie kans. De Capanna techniek is bijzonder geschikt na tumorresectie bij personen met een lage leeftijd.

Concluderend, het gebruik van VBG blijft tot op heden de gouden standaard voor de reconstructie van grote segmentale bot defecten in de onderste extremiteit. Ter bescherming van mechanische belasting kan het beste de VBG gecombineerd worden met een CBA wanneer het gaat om een jonge patiënt met een tumor indicatie. De primaire bot genezing is beter wanneer een VBG gebruikt wordt dan een CBA alleen. Het gebruik van gevasculariseerd bot is gelimiteerd in het gebruik door de slechts enkele beschikbare botten die “gemist” kunnen worden in het menselijk lichaam zonder functie in te leveren. Daarnaast is zijn VBG geassocieerd met complicaties die kunnen ontstaan waar het donor bot wordt geogst.

Deel II:

In het tweede deel van deze promotie, ligt de focus op een nieuwe alternatieve reconstructie methode; namelijk het gebruik van gevasculariseerde allogene bot transplantaties (VCAs). Hierbij wordt een geselecteerd bot van een menselijke donor wordt geogst en vervolgens gevasculariseerd aangesloten in een menselijke ontvanger. Deze donor is tevens vaak ook nier, hart, long en lever donor. Deze alternatieve methode is slechts in enkele gevallen ook daadwerkelijk in mensen verricht. Gevasculariseerde allotransplantaties zijn gelimiteerd door het gebruik van levenslange immunosuppressiva zoals benodigd bij elke vorm van orgaantransplantatie. In het microvasculair onderzoekslaboratorium van de Mayo Clinic is een nieuwe methode ontwikkeld die weefsel vitaliteit van een levend bot allotransplantaat behoudt zonder levenslange immunosuppressiva. Deze nieuwe methode van levende bot allotransplantaties combineert het microchirurgisch aansluiten van de allogene bloedvaten in de ontvanger met het aanleggen van een tweede autologe circulatie. Hierbij zijn slechts twee weken immunosuppressiva benodigd. De tweede autologe circulatie wordt aangelegd door een arterioveneuze (AV) bundel te implanteren in het allotransplantaat. De werkzaamheid van deze nieuwe methode van transplanteren is reeds in kleine diersmodellen aangetoond. Als hypothese stelde wij dat de weefsel vitaliteit in bot VCAs in een groot diersmodel kan worden behouden door chirurgisch geïnduceerde neo-angiogenese (AV-bundel implantatie) met kortdurende immunosuppressiva. Daarnaast verwachten wij dat de nieuwe bot formatie in het allotransplantaat het resultaat is van autologe bot cellen die migreren van de ontvanger in het transplantaat. Hierdoor zal over de tijd het transplantaat worden gerepopuleerd door cellen van de ontvanger.

In Hoofdstuk 4, werd gedemonstreerd dat wij succesvol een defect in een varkens tibia kunnen reconstrueren door middel van een VCA van een donor varken. Hierbij transplanteerde wij van een manlijke donor naar een vrouwelijke ontvanger. De varkens waren gepaard voor het bloedgroep type, echter was er een opzettelijk groot verschil in histocompatibiliteit (Swine Leukocyte Antigen mismatch). Tijdens de transplantatie implanteerde wij de AV-bundel in de intramedullaire ruimte van het transplantaat om autologe neo-angiogenese te induceren. Om te evalueren of AV-bundel implantatie daadwerkelijk voor behoudt van weefsel vitaliteit zorgt, verdeelde wij de varkens in twee groepen. In groep 1 implanteerde bij een patente AV-bundel en in groep 2 implanteerde wij een proximaal geligeerde AV-bundel als controlegroep. In beide groepen werd de allogene circulatie hersteld en werd er interne fixatie van het transplantaat verkregen. Met succes transplanteerde wij 12 gevasculariseerde allogene tibia segmenten (VCAs). Het allotransplantaat bleek bloedvoorziening te krijgen uit de allogene vaatsteel in de eerste 4-6 weken. Dit leidde vervolgens tot significant nieuwe botformatie welke heeft geresulteerd tot botgenezing. Chirurgisch geïnduceerde neo-angiogenese had geen tegenstrijdig effect op de biomechanische eigenschappen van het allotransplantaat. Wel bleek de neo-angiogenese een positief effect te hebben op de bot remodelering eigenschappen van het allotransplantaat, ondanks trombose van de allogene vaatsteel in de eerste 4-6 weken. Het effect van chirurgisch geïnduceerde neo-angiogenese op weefsel vitaliteit en genetische expressie werd verder geëvalueerd in Hoofdstuk 5. Uit dit hoofdstuk bleek dat AV-bundel implantatie in groep 1, leidde tot significant meer ingroei van bloedvaten in het allotransplantaat 20 weken na transplantatie. Dit leidde vervolgens tot verbeterde osteocyt (bot cellen) scores dus beter behouden weefsel vitaliteit. In groep 1 werd ook significant meer expressie van endothelial growth factor like-6 (EGFL6) gevonden welke positief gecorreleerd was aan het gemeten vaatvolume in het allotransplantaat.

Om het mechanisme te bestuderen die ten grondslag liggen aan de succesvolle VCAs getransplanteerd met onze nieuwe methode, wordt in Hoofdstuk 6 een studie verricht die het geslacht van de cellen in de VCA kan herleiden. Gezien wij transplantaties verrichte van manlijk geslacht naar vrouwelijk geslacht, kunnen wij de omvang van een gemixt chimerisme bepalen in de bot allotransplantaten. Om dit te doen, analyseerde wij DNA en RNA middels RT-qPCR. Middels deze PCR-techniek, kunnen we de relatieve verhouding van het seks-bepalende gebied Y (SRY)-gen (specifiek alleen voor het mannelijke DNA en RNA) berekenen ten opzichte van een huishoudster gen (een gen wat in alle cellen voorkomt). Wij verkregen DNA en RNA uit specifieke gebieden van het allotransplantaat middels Laser Capture Microdissectie (LCM). Met deze technieken, kunnen we heel specifiek onderzoeken van welke geslacht de nieuwe bot formatie is. We vonden dat nieuwe bot formatie voornamelijk afkomstig is van vrouwelijke cellen die waren gemigreerd van de ontvanger in het allotransplantaat. Daarnaast vonden we dat sommige mannelijke cellen de transplantatie hadden overleefd. Manlijke cellen waren niet geëmigreerd naar andere organen zoals lever en milt. Over de tijd zal het allotransplantaat dus worden gerepopuleerd door autologe (vrouwelijke) cellen.

In ons allotransplantatie model gebruikte wij slechts twee weken immunosuppressiva. Na het stoppen van de immunosuppressiva zou men verwachten dat het allotransplantaat wordt afgestoten door de ontvanger. In Hoofdstuk 7, onderzochten we de systemische immunologische reactie en het effect van AV-bundel implantatie op de lokale immuunreactie

na allotransplantatie. Het resultaat van het stoppen van immunosuppressiva in ons diermodel leidde ertoe dat de allogene vaatsteel na 4-6 weken getromboseerd was, zoals in hoofdstuk 4 beschreven. Histologische evaluatie van de allogene vaatsteel, 20 weken na transplantatie, liet zien dat dit het gevolg was van intima hyperplasie. Intima hyperplasie is een fenomeen wat we vaker zien in de wand van bloedvaten als gevolge van een immunologische afstotingreactie. Trombose van de allogene vaatsteel was een verwacht effect. Normaliter zou dit tot verlies van weefsel vitaliteit leiden. Echter in deze studie, vormt de AV bundel een nieuwe circulatie tijdens de immunosuppressiva periode. Hierdoor vonden wij dat door AV-bundel implantatie weefsel vitaliteit was behouden. Tevens werd er minder fibrose en inflammatie gevonden als gevolg van AV-bundel implantatie. Histologisch onderzoek van de AV-bundel zelf liet geen intima hypertrofie zien. Middels regelmatige bloedafnames tijdens de 20-weeken overleving konden we de systemische immunologische reactie op transplantatie monitoren. De gemeten witte bloedcellen, rode bloedcellen, lymfocyten, monocyten, eosinofiele, neutrofiële en cytokinen lieten geen tegenstrijdig effect zien als gevolg van transplantatie.

Middels dit proefschrift hebben wij in een groot preklinisch diermodel aangetoond dat onze nieuwe methode van allotransplantatie een significant voordeel lijkt te hebben vergeleken met conventionele allotransplantatie procedures. De experimentele uitkomsten besproken in hoofdstuk 4-7 zijn consistent met de uitkomsten gevonden in klein diermodellen die dezelfde gevasculariseerde bot allotransplantaties uitvoerde met gebruik de nieuwe transplantatie methode [12-18]. Het varkens model heeft specifieke voordelen die VCA-onderzoek vertaalbaar maken naar de menselijke kliniek. De anatomie, grootte, fysiologie en immunologie van varkens is onderzoekers welbekend en lijkt op die van de mens. Het door ons gebruikte varkens model in dit proefschrift heeft aangetoond van grootte waarde te zijn voor VCA-onderzoek. De data die wordt verkregen met dit varkens model blijkt consistent, reproduceerbaar en vertaalbaar naar de menselijke kliniek. Gevasculariseerde bot allotransplantaties hebben om deze reden de potentie voor toekomstige toepassing in de menselijk kliniek wanneer gecombineerd met onze nieuwe transplantatie methode.

Deel III:

In het derde deel van dit proefschrift hebben wij geprobeerd om de nieuwe transplantatie methode te extrapoleren naar gehele gewricht transplantaties. Hiervoor hebben wij een nieuw groot diersmodel ontwikkeld waarin wij een groot knie defect reconstrueren met een gevasculariseerde gehele knie van een donor, waarbij slechts twee weken immunosuppressiva werd gebruikt. Hierbij werd getracht weefsel vitaliteit trachten te behouden door autologe AV-bundel implantatie en een gesteelde gracilis spier lap (chirurgische neo-angiogenese). Wij stelde de hypothese dat chirurgisch geïnduceerde neo-angiogenese weefsel vitaliteit, genezing en gewrichtsfunctie behoudt met kortdurende immunosuppressiva.

In Hoofdstuk 8 beschrijven wij een nieuwe werkwijze van gevasculariseerde gewrichtstransplantatie met chirurgisch geïnduceerde neo-angiogenese en kortdurende immunosuppressiva, waarbij een anatomie en pilotstudie is verricht. Een gewricht bestaat uit meerdere type weefsels zoals bot, kraakbeen, ligamenten, kapsel en bloedvaten. Aangezien de bloedvoorziening hiervan significant meer complex is vergeleken met een bot allotransplantaat. Hebben wij getracht naast AV-bundel implantatie het gehele gewrichtstransplantaat te bedekken met een autologe gesteelde gracilis spier lap om ook vanuit de periferie neo-angiogenese te stimuleren in de verschillende type weefsels. In de anatomie en pilotstudie blijkt dat het chirurgisch technisch haalbaar is om op deze manier gehele gewrichtstransplantaties te verrichten. Echter, de complexiteit, kosten en de complicaties die wij hebben ervaren resulteerde in vroegtijdige terminatie van het experiment. Wij concluderen dan ook dat gehele gewrichtstransplantaties een extreem complexe chirurgische procedure is, waarin het diersmodel nog verder moet worden ontwikkeld. Daarnaast zal de postoperatieve zorg moeten worden verbeterd om complicaties te voorkomen.

Conclusie

In de toekomst verwachten wij dat gevasculariseerde allotransplantaties meer toegankelijk en geaccepteerd gaan worden. Alhoewel er al klinische ervaring bestaat met gevasculariseerde hand, gezicht en buikwand allotransplantaties doormiddel van kennis verkregen uit orgaantransplantaties. Zou toekomstig VCA-onderzoek zich moeten focussen op VCA-transplantatie veiligheid, transplantatie protocollen aanvullend op orgaantransplantatie, en veelzijdigheid van de te transplanteren bot segmenten. Daarnaast zou in verder experimenteel dieronderzoek de mogelijkheden van gehele gewrichtstransplantatie verder moeten worden onderzocht. Met de huidige kennis verkregen uit voorgaand VCA-onderzoek gecombineerd met onze nieuwe methode van VCA-transplantaties bieden wij een nieuwe optie voor de reconstructie van grote segmentale bot defecten. Hierbij is het van essentieel belang te erkennen dat reconstructieve chirurgie geen levensverlengende chirurgie is. Daarom moeten de risico's geassocieerde met reconstructieve chirurgie zo klein mogelijk blijven. Do no further harm.

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cells
AV	Arteriovenous
B.pm	Bone Parameter
BFR	Bone Formation Rate
BGLAP	Bone Gamma-Carboxyglutamate Protein (osteocalcin)
BMA	Bone Micro Architecture
BS	Bone Surface
CBA	Cryopreserved Bone Allografts
CD	Cluster Differentiation
cDNA	Copy DNA
CI	Confidence Interval
CT	Computed Tomography
CTSA	Center for Translational Sciences Activities
CTSK	Cathepsin K
dLS	Double Labeled Surface
DNA	Deoxyribonucleic Acid
EGFL	Epidermal growth factor like 6
FFPE	Formalin Fixed Paraffin Embedded
GAPDH	Glyceraldehyde 3-Phosphate dehydrogenase
gDNA	Genomic DNA
GvHD	Graft versus Host Disease
HIF1A	Hypoxia Induced Factor 1a
HLA	Human Leukocyt Antigen
IACUC	Institutional Animal Care and Use Committee
IFN	Interferon-Gamma
IL	Interleukin
IM	Intra Muscular
IM-nail	Intra-medullary nail
IS	Immunosuppression
IV	Intra Venous
LCM	Laser Capture Microdissection
LCP	Locking Compression Plate
LS	Labeled Surface
mRNA	Messenger RNA
MS	Mineralizing Surface
MSTS	Musculoskeletal Tumor Society
n	Number
N.Lac	Number of Lacunae
N.Ot	Number of osteocytes
NIDI	Normalized Indentation Distance
NIH	National Institute of Health
OPG	Osteoprotegerin
p	probability
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEN	Polyethylene Naphthalate
RANKL	Receptor Activator Nuclear Factor κ B Ligand

RNA	Ribonucleic Acid
RPI	Reference Point Indentation
RPL-4	Ribosomal Protein L-4
RT-qPCR	Reverse Transcript quantitative Polymerase Chain Reaction
SD	Standard deviation
SLA	Swine Leukocyt Antigen
sLS	Singe Labeled Surface
SRY	Sex determing Region Y- Chromosome
SRY-e	Sex determing Region Y- Chromosome exonic
TID	Total Indentation Distance
TNF	Tumor Necrosis Factor
TNRSF11	Tumor Necrosis Factor Superfamily 11-a/RANKL
TNRSF11B	Tumor Necrosis Factor Superfamily 11-b / OPG
VBG	Vascularized Bone Grafts
VCA	Vascularized Composite Allotransplantation
VEGFA	Vascular endothelial growth factor A

Units

ml	Microliter
cm	Centimeter
d	Day
kg	Kilogram
kV	Kilo Volts
mcg	Microgram
mg	Milligram
ml	Milliliter
Mpa	Elastic Modulus
N	Newton
ng	Nanogram
s	Second
uA	Micro Ampères
um	Micrometer

CURRICULUM VITAE

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Extracurricular activities

- 2015 (June/Augustus) Plastic, Reconstructive and Hand Surgery
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Extracurricular clinical and scientific internship
- 2013-2014 General Surgery
Leiden University Medical Center (LUMC), Leiden, The Netherlands
Researcher Transplantation Surgery: role of ischemia and reperfusion injury
- 2010-2013 General Surgery
Leiden University Medical Center (LUMC), Leiden, The Netherlands
Researcher Trauma Surgery: Zuggurtung fixation Olecranon fractures
-

PHD PORTFOLIO

Scientific talks

- 2020 (Jan) Outcomes of vascularized bone allotransplantation with surgical induced autogenous angiogenesis and short-term immunosuppression in a large animal model: bone healing, pedicle patency, viability and remodeling due to recipient derived neo-angiogenesis, ASRM, Marriot Fort-Laurendale FL, USA
- 2019 (Nov) Zijn gevasculariseerde bot en gewricht allotransplantaties de toekomst? Bi-annual meeting, Dutch society for Plastic surgery, NVPC, Leeuwarden, The Netherlands
- 2019 (April) Gevasculariseerde allogene bot transplantaties een nieuwe reconstructie methode, *Bi-annual meeting, Dutch society for Plastic surgery, NVPC, Amsterdam, The Netherlands*
- 2018 (Oct) Living bone allotransplantation: a novel reconstruction method, Patrick J. Kelly Resident and Fellow Research competition, Mayo Clinic, 29-10-2018 (Winner)
- 2017 (May) Living bone allotransplantation, Wetenschapsdag, VUmc, 19-05-2017

Congresses attended

- 2020 (Jan) Annual Meeting ASRM 2020, American Society for Reconstructive Microsurgery, Fort-Laurendale, FL, USA
- 2019 (Nov) Bi-annual meeting, Dutch society for Plastic surgery, NVPC, Leeuwarden, The Netherlands
- 2019 (May) Bi-annual meeting, Dutch society for Plastic surgery, NVPC, Amsterdam, The Netherlands
- 2019 (March) AMS annual meeting, Amsterdam, The Netherlands
- 2018 (October) Annual Meeting ASSH 2018, American Society for Surgery of the Hand, Boston MA, USA
- 2018 (October) Grand Rounds Orthopedic Surgery: Bone Sarcomas: kids, dogs, and future, Mayo Clinic, Rochester MN, USA
-

2018 (September)	American Society for Surgery of the Hand (ASSH), annual meeting 2018, Boston, USA
2018 (May)	Grand Rounds Orthopedic Surgery: Orthoplastic reconstruction-principles, Mayo Clinic, Rochester MN, USA
2018 (March)	Grand Rounds Plastic Surgery: Facial reanimation, Mayo Clinic, Rochester MN, USA
2018 (March)	Grand Rounds Plastic Surgery: Case presentations Hand /general reconstruction, Mayo Clinic, Rochester MN, USA
2017-2018	Weekly Hand Conference, Mayo Clinic, Rochester MN, USA
2017 (May)	Bi-annual meeting, Dutch society for Plastic surgery, NVPC, Amsterdam, The Netherlands
2017 (April)	Bi-annual meeting, Dutch society for Plastic surgery, NVPC, Rotterdam, The Netherlands

Courses/certificates

2018 (October)	TriMed resident seminar, fractures of the hand and wrist, San Diego, USA
2018 (October)	TriMed, Hand and Wrist course fellows, Mayo Clinic, Rochester MN, USA
2018 (September)	Research methods, ASSH, Boston, USA
2018 (April)	Utilizing statistics in clinical research; Mayo Clinic, Rochester MN, USA
2018 (February)	Statistics clinical research; Mayo Clinic, Rochester MN, USA
2017 (October)	Case series and entrepreneurship; Mayo Clinic, Rochester MN, USA
2017 (October)	TriMed resident seminar hand and wrist; Charlotte NC, USA
2017 (September)	Microsurgery course; Mayo Clinic, Rochester MN, USA
2017 (August)	Research integrity and compliance training; Mayo Clinic, Rochester MN, USA
2017 (August)	Care and handling of laboratorial Swine and Rats; Mayo Clinic, Rochester MN, USA
2017 (August)	Infection prevention and control & blood pathogens, Mayo Clinic, Rochester MN, USA
2017 (August)	Animal allergies and infectious control methods; Mayo Clinic, Rochester MN, USA
2017 (August)	Conflict of interest tutorial; Mayo Clinic, Rochester MN, USA
2017 (August)	Care and use of animal research; Mayo Clinic, Rochester MN, USA
2017 (June)	Microsurgery course; Erasmus MC, Rotterdam, The Netherlands
2016 (October)	ATLS-certification; Eindhoven, The Netherlands
2016 (August)	ABC-cursus + BLS-cursus; Hagaziekenhuis, The Hague, The Netherlands
Award:	Jowsey Research Fellow Award 2018, Orthopedic Surgery, Mayo Clinic, USA

Other Publication

1. Ned Tijdschr Geneeskd. 2014;158:A7322.

A woman with chronic infection of the upper legs.

Houben RH, Hamdy NA, van Dissel JT, Schipper IB.

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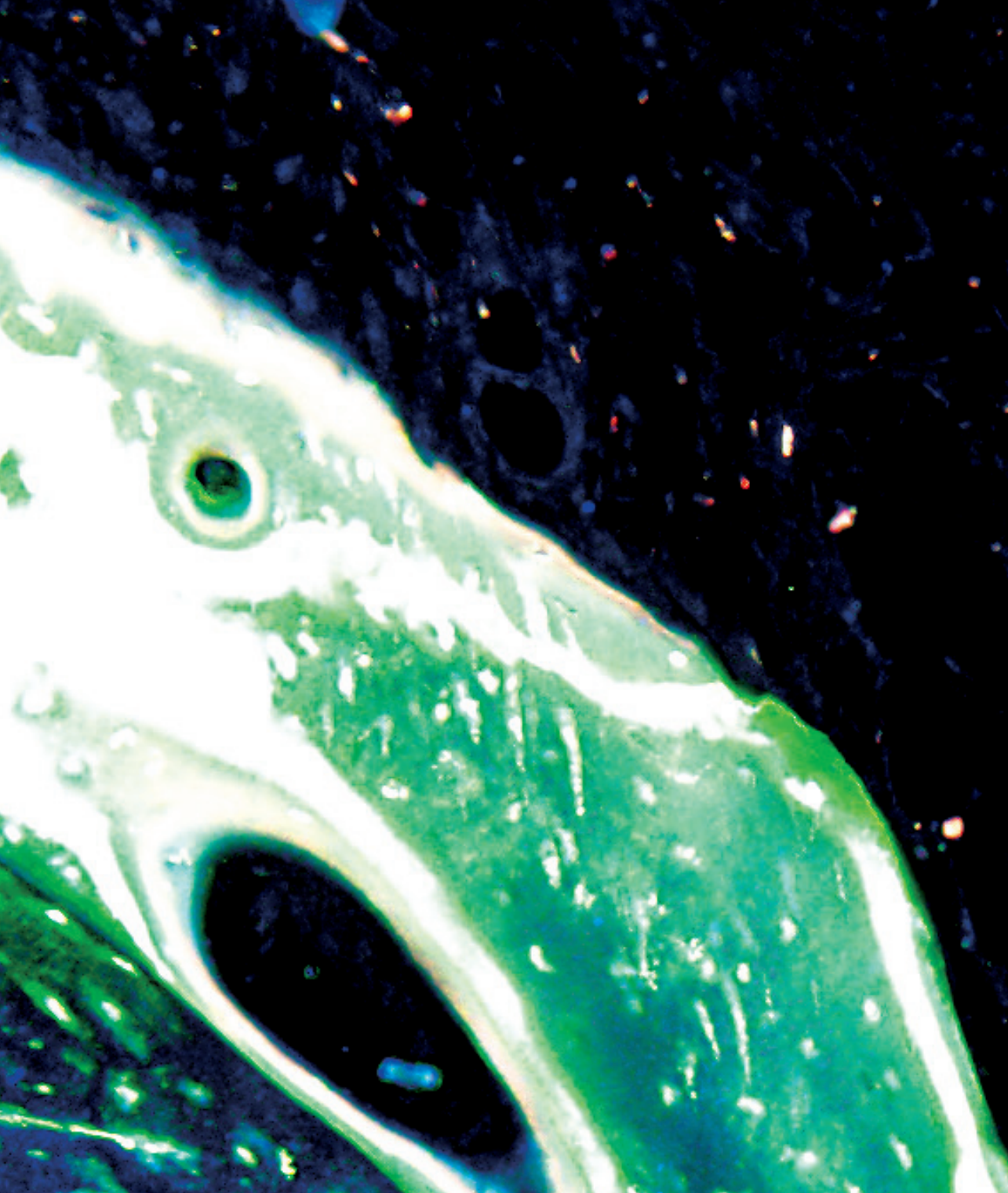
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