Chapter 6
Pathophysiology of Volumetric Muscle Loss and Targets for Regenerative Rehabilitation

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Abstract Orthopedic extremity trauma is a major problem resulting in both long-term functional disability and substantial medical cost in various populations. One such injury is volumetric muscle loss (VML), which is clinically identified as a chronic and irrecoverable loss of skeletal muscle tissue resulting in functional impairments. VML is coupled with clinical outcomes related to long-term dysfunction, reduced mobility and physical activity, comorbidities, and often delayed amputation. The objectives for this chapter are to (1) summarize known pathophysiologies associated with VML injury and tools for their clinical and experimental assessment, (2) review current rehabilitation and regenerative medicine strategies for VML injury, and (3) explore the evidence supporting regenerative rehabilitation as a strategy to maximize functional recovery of the VML-injured limb and discuss knowledge gaps and areas for scientific advancements.

Keywords Muscle strength · Mitochondria · Physical therapy · Exercise training · Muscle adaptation

6.1 Introduction

The clinically accepted definition for volumetric muscle loss (VML) injury is "the traumatic or surgical loss of skeletal muscle with resultant functional impairment" (Grogan and Hsu 2011). Traumatic muscle injuries like VML often result from blunt

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force trauma in which an object strikes the body, from penetrating traumas in which an object pierces the body creating an open wound, or controlled, surgical trauma in which an object is removed from the body. Although traumatic injuries often cross tissue boundaries such as bone, nerve, vascular, tendinous, ligamentous, and/or cartilaginous, this chapter focuses on skeletal muscle pathology and regenerative rehabilitation strategies to improve skeletal muscle function. Skeletal muscle makes up ~40% of the body's mass and contributes to locomotion, thermoregulation, and whole-body metabolism. Any loss of skeletal muscle function can negatively affect mobility and increase an individual's risk of all-cause morbidity and mortality.

VML injury can result secondary to any of the 150,000 open fractures or 30,000 gunshot wounds (trauma), 36,000 chainsaw accidents (industrial/farm), and 13,000 soft-tissue sarcomas (cancer) that occur annually in the USA. Approximately 77% of all military casualties are musculoskeletal injuries, many that have a component of debilitating VML injuries (Cross et al. 2011; Owens et al. 2007; Owens et al. 2008). Data from the US trauma centers indicate that two-thirds of traumatic injuries occur to extremities with 32% and 40% to the upper and lower extremities, respectively. Collectively, the US economic burden related to trauma and injury is $400 billion yearly (Seifert 2007).

This chapter (1) describes the pathophysiology of VML injury, (2) discusses common VML study outcome measurements, (3) reviews rehabilitation and regenerative medicine approaches to improve VML, and (4) highlights the need for regenerative rehabilitation studies with functionally relevant outcome measurements to advance evidence-based approaches for VML.

6.2 Current Standards of Care

The current practices for definitive care following VML injury and orthopedic injuries involving concomitant VML injury can be generalized into three phases: (1) immediate field care, (2) acute surgical care, and (3) short-term rehabilitative care (Saunders and Rose 2021; Dolan et al. 2021). Standardization within these phases is complicated by the site of the injury and poly-traumatic nature of the injury that can include damage to the bone, muscle, skin, nerve, and/or vascular network. Prioritization of care among the phases starts with patient survival, minimizing infection risk, limb salvage, and finally tissue rehabilitation. Immediate field care often takes place at the site of injury (e.g., field of play, battlefield) and involves fixation of the limb for emergency transport, field dressing to minimize infection risk, and possible tourniquets to reduce blood loss from the injury. These practices prioritize patient survival. Acute surgical care involves surgical procedures and postoperative procedures conducted at a trauma center. Standard practices include blood transfusion, bone union, muscle flaps, nerve allografts, and limb amputation if required for survival (e.g., infection). Evidence of rehabilitative approaches applied in the intensive care unit immediately following acute surgical care is nonexistent, although could involve neuromuscular stimulation and/or mobilization. Short-term

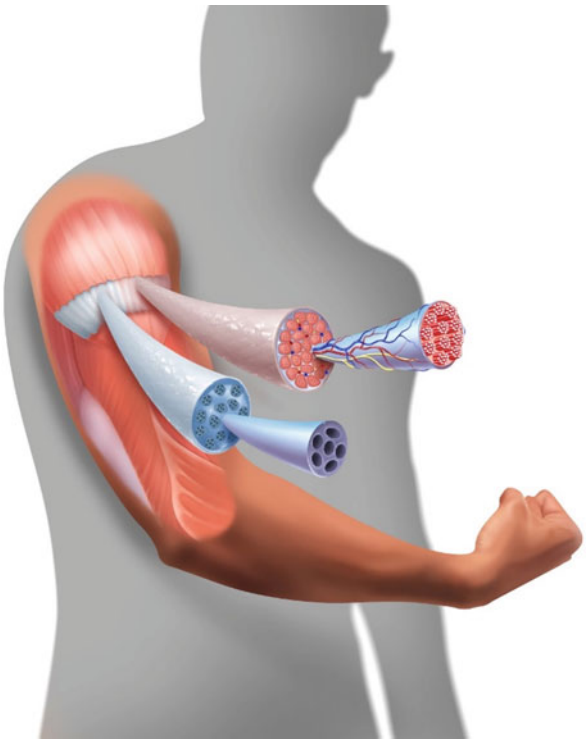

Fig. 6.1 The goal of regenerative rehabilitation. Regenerative rehabilitation seeks to leverage a regenerative medicine technology (exemplified here as a biomaterial-based muscle fascicle) with validated rehabilitation approaches to maximize muscle restoration and functional capacity. Ideally, a regenerative rehabilitation approach has a greater effectiveness than either regenerative medicine or rehabilitation alone. This is exemplified here by a biomaterial-based muscle fascicle with vascularized (yellow) and perfused (red/vessels) muscle fibers

rehabilitative care often prioritizes task rehabilitation, e.g., regaining mobility or ability to navigate stairs, as opposed to functional rehabilitation (i.e., improving strength) due to a lack of evidence showing robust improvements in muscle function with current strategies. To address this challenge, there is an urgent need to identify new or existing strategies in combination that can bolster functional recovery. One idea is to explore regenerative rehabilitation approaches.

Regenerative rehabilitation was broadly defined in Chap. 1, so here a more specific definition will be advanced for VML injury. *Regenerative rehabilitation for VML injury is a focused effort to improve skeletal muscle quality and overall muscle function and advance mobility and quality of life for the patient by integrating regenerative technologies with rehabilitation clinical practices* (Fig. 6.1). For the VML-injured patient, any functional gain beyond those achieved with rehabilitation are expected to have a considerable impact on quality of life and as such regenerative rehabilitation may be an ideal tool to accomplish this goal.

6.3 Pathophysiology

In this section, we highlight primary pathologies associated with VML injuries, and then summarize outcome measurement commonly used to evaluate the effectiveness of an intervention. VML injuries are idiosyncratic and any one injury may not involve all of the pathologies discussed below. Additionally, investigators should consider the pros and cons of each technique in relation to their study design questions and anticipated outcomes. Throughout this section we will reference Fig. 6.2 that provides examples of common pathologies after VML injuries at four different regions of the VML-injured muscle. These four regions include (1) the defect, representing the area in which a volume of muscle was removed; (2) the border, representing the tissue layer between the defect and remaining muscle; (3) the near defect, representing the remaining muscle in close proximity to the defect; and (4) the distant to defect, representing the remaining muscle far enough from the defect area that it was not injured by the initial VML injury. The pathologies taking place at these four muscle regions can all contribute to the long-lasting functional deficits observed after VML injury and the lack of full muscle restoration, and are in contrast to the full muscle recovery capable after more common contraction-induced muscle injuries.

6.3.1 Loss of Muscle Function

Skeletal muscle has many roles, perhaps the most important is to facilitate movement. Thus, the drastic loss of muscle function following a VML injury is considered the most consequential (Corona et al. 2016). Clinically, individuals that have some form of VML injury display a wide spectrum of functional deficits including strength loss, limited range of motion, and muscle stiffness. The gold standard for quantifying the extent of an injury or the efficacy of a regenerative or rehabilitative intervention is by testing the functional capacity of the muscle. Follow-up assessments also show conflicting results related to the ability of the muscle to restore function several months post-injury. In some VML injuries, the limb may be completely void of any initial functional strength or intervention-induced strength gains, while other VML injuries, though limited, can show promise for a restoration of function over time. Overall, the loss of function is typically disproportionally greater than the size of muscle removed (Corona et al. 2016). The most unfortunate finding from clinical work is that muscle function may actually decline chronically, despite the use of rehabilitative and/or regenerative interventions (Mase et al. 2010; Tanaka et al. 2017; Sicari et al. 2014; Gentile et al. 2014; Garg et al. 2015).

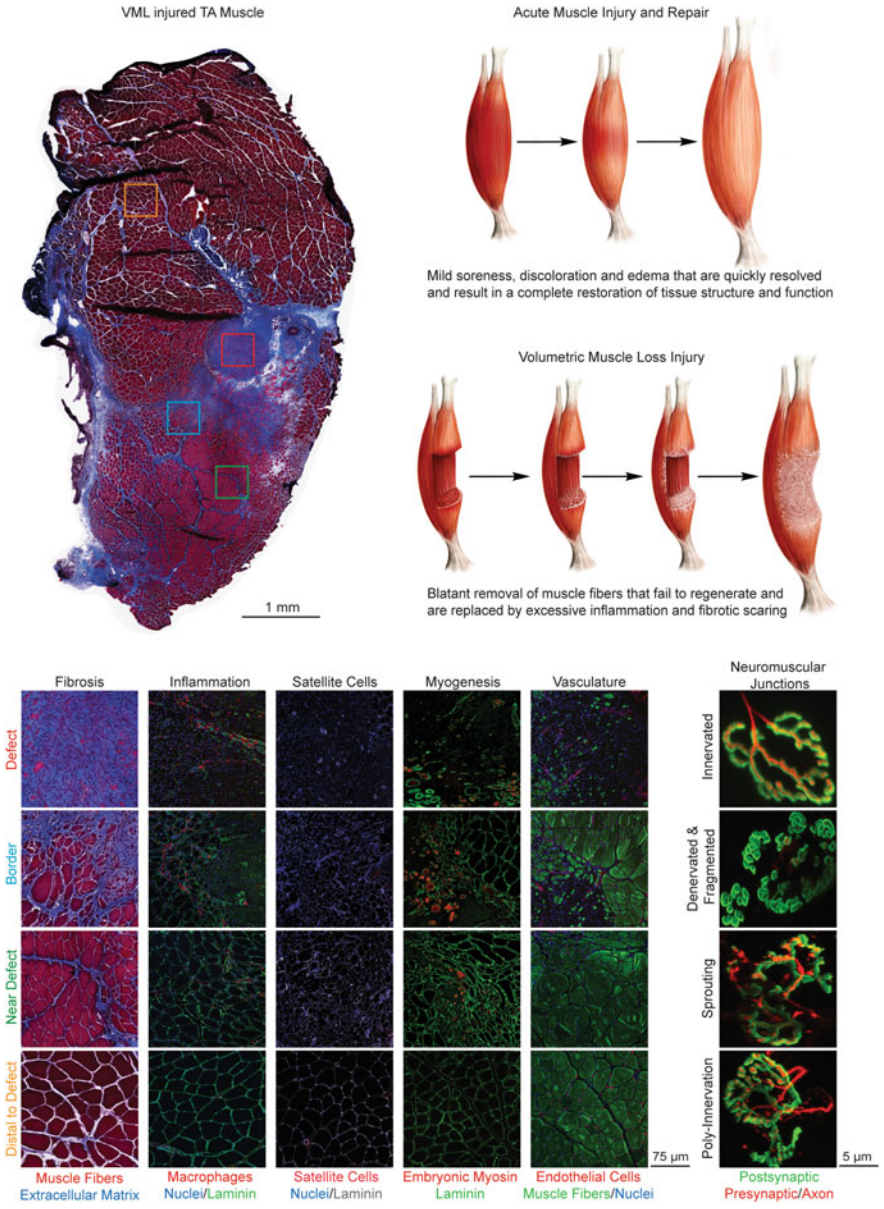

Fig. 6.2 Pathophysiology of volumetric muscle loss injury. Acute skeletal muscle injuries (e.g., strains, contusions, and exercise induced) follow a canonical sequence of cellular events that typically result in the successful restoration of the damaged tissue to its previously uninjured and functional state. Conversely, VML injuries are plagued with an irregular pathologic response that overwhelms the remaining tissue. The VML pathology is greatest in and around the defect area and gradually declines at the more distal regions of the injured tissue. Characteristics include excessive fibrosis, persistent and irregular inflammation, poor satellite cell migration, and activation, limited formation of new muscle fibers, changes in vascularity, and dysregulation of the remaining neuromuscular junctions. Ultimately, VML-injured muscle fails to recover the structural and function components of healthy skeletal muscle tissue, often resulting in long-term disability. The

6.3.1.1 Experimental and/or Clinical Outcome Measurements

Clinically, muscle function is measured using video photography, a goniometer, and a biodex dynamometer for assessing gait, range of motion, and functional strength, respectively. The standardized use of these modalities allows for continual evaluation of VML strength for understanding long-term complications and/or progress of a physical therapy. It also serves as a mechanism to facilitate coordinated and individualized care by the medical staff.

The development and standardization of VML injury models and equipment have advanced preclinical VML research (Garg et al. 2015; Greising et al. 2019). For example, VML injuries in the tibialis anterior (TA), gastrocnemius, quadriceps, and latissimus dorsi muscles of animals have been developed to allow neuromuscular strength testing (Greising et al. 2019). While it is possible to measure grip strength and forced treadmill running, the use of a functional muscle testing system provides the most accurate results for testing force generation of specific muscles of interest and can be performed in vivo, in situ, or in vitro (e.g., Quarta et al. 2017; Corona et al. 2013a; Corona et al. 2013b). As muscles vary in size and strength, absolute strength is often normalized to body or muscle mass. Importantly, the use of a functional muscle testing system with preclinical VML models enables specific examination of various rehabilitation and regenerative medicine paradigms that are not yet suitable clinical trials (e.g., a new biological scaffold).

An emerging method for measuring muscle function is the biomechanical assessment of ambulation and gait kinematics. Two different approaches have been used to accomplish this, both include the use of videography, and it is recommended that treadmill acclimation be performed prior to testing (Dienes et al. 2019; Quarta et al. 2017). In the first approach, reflective markers are placed on bony landmarks that correspond with the pelvis, femur, tibia, and foot. Animals are then placed on a treadmill and a multi-camera setup is used to identify and record the reflective markers with their corresponding joint angles and segment lengths for gait kinematic analysis. This method has been used to detect differences in gait between healthy and VML-injured limbs and can be used in combination with force data or separately to evaluate muscle function (Dienes et al. 2019). In the second approach, a video imaging system captures gait kinematics from the lateral and ventral perspective in rodents using a transparent treadmill belt (e.g., Digigate; Mouse Specifics Inc.). This method provides a unique view of the animal's gait while also generating digital paw prints that allow for the identification of gait abnormalities (Quarta et al. 2017). The data collected from both of these approaches can be used to assess strength, balance, gait, and coordination, making it a useful tool for evaluating the effectiveness of a regenerative and/or rehabilitative intervention.

Fig. 6.2 (continued) images in this figure are representative of a VML injury at different locations in a rat tibialis anterior muscle 21 days following the surgical removal of a full thickness, 6 mm biopsy punch. Cell identification markers used in this figure include the colocalization of nuclei (DAPI) with $CD68^+$ macrophages, $Pax7^+$ satellite cells, or $CD146^+$ endothelial cells

The drastic loss of muscle function brought on by VML is thought to be mostly attributed to the abrupt removal of muscle fibers. Thus, the majority of regenerative interventions focus on stimulating de novo myofiber formation using biomimetic scaffolds and various stem cell interventions. However, after accounting for the mass of tissue or the number of fibers removed and/or damaged due to the VML injury, there remains a glaring discrepancy in strength loss that is unaccounted for, possibly a result of metabolic deficiencies, satellite cell dysfunction, denervation, impaired excitation-contraction coupling, extensive inflammation, connective tissue accumulation, and/or alterations in the length tension relationship of the myosin/actin cross-bridge. As the study of VML injury is a relatively young and growing field, gaps currently exist in understanding the long-term consequences of VML injury. As such, pathologies moving forward are supported by the literature, but it is entirely possible that this list will grow along with advanced knowledge of the VML injury.

6.3.2 Loss of Metabolic Function

The effects of VML injuries are not constrained to mobility deficits. Loss of functional muscle fibers is analogous to a stark reduction of metabolically active cells that, for example, contribute to basal metabolic rate and circulating glucose levels. At a cellular level, skeletal muscle's metabolic efficiency is regulated by the mitochondria. Within this organelle, several factors contribute to this metabolism, including enzyme kinetics of the components of the electron transport chain, oxygen consumption, and mitochondrial membrane polarization. Oxidative capacity has been shown to decrease by roughly 25% in VML-injured muscle when compared to uninjured muscle (Greising et al. 2018; Southern et al. 2019). This could have potential whole-body implications as VML-injured mice are reported to have a lower overall metabolic rate despite similar levels of physical activity (Dalske et al. 2021). Such whole-body changes can lead to metabolic deficits associated with an increased likelihood of developing diabetes, cardiovascular disease, and metabolic syndrome. The muscle-specific and whole-body characterization of metabolic changes after VML is relatively new and there is a large knowledge gap relating to clinical manifestations and cellular mechanisms.

6.3.2.1 Experimental and/or Clinical Outcome Measurements

Muscle metabolism is orchestrated, in large part, by a collection of enzymes found within the mitochondria that aid in the process of breaking down pyruvate to create ATP. Briefly, pyruvate dehydrogenase oxidizes pyruvate to create acetyl-CoA by way of the reduction of NAD+ to NADH. The action of the enzyme citrate synthase condenses acetyl-CoA and oxaloacetate into a six-carbon molecule, citrate. Citrate will undergo stepwise oxidation at the hands of the enzymes isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and succinate dehydrogenase, among others,

until oxaloacetate is recovered. Rates of activity from these enzymes have been successfully measured in skeletal muscle tissue previously and are prime targets to monitor the disease progression and subsequent recovery of skeletal muscle in models of VML injury (Corona et al. 2018a; Southern et al. 2019; Chao et al. 2019).

Products from the tri-carboxylic cycle are used in the mitochondria to further produce ATP through oxidative phosphorylation. Here, the reduction of NADH and FADH2 will produce electrons that will be shuttled between the respiratory complexes and H+ ions that are pumped into the intermembrane space, with oxygen acting as the final electron acceptor. The shuttling of electrons through the respiratory complexes allows for more H+ to be pumped into the intermembrane space and for additional electrons to be shuttled down the pathway. Therefore, a decrease in oxygen consumption, the final step in the process, can point toward deficiencies in skeletal muscle metabolism. To this point, studies have reported a decrease in oxygen consumption at State III in VML-injured muscle compared to uninjured controls (Greising et al. 2018; Southern et al. 2019; Chao et al. 2019).

To date, studies have relied on the substrate-uncoupler-inhibitor titration (SUIT) method in high-resolution respirometry to determine oxygen consumption. Substrates that feed each complex within the electron transport chain are added into a closed system with a known amount of oxygen. Rate of oxygen consumption at each titration is compared to a control to identify changes in mitochondrial function. While this method offers the ability to observe mitochondrial deficiencies on a complex-by-complex basis, it also paints an idealistic picture of oxygen consumption. This is due to the fact that substrates provided into the experimental system are at supra-physiological concentrations where you assume the limiting factor is the materials needed to balance the ADP/ATP ratio. A more recent respirometry method, known as the creatine kinase clamp (CK clamp), utilizes known facts about ADP/ATP and Cr/PCr ratios during rest and exercise to calculate energetic demand and the tissue's ability to adapt when that energetic demand increases or decreases (Fisher-Wellman et al. 2018). In this assay, the ADP/ATP ratio is maintained constant, while titrations of phosphocreatine lower energetic demand, and, likewise, oxygen consumption will also decrease. Other indirect measures for the activity if CK in skeletal muscle following VML have also been achieved through in vitro systems (Li et al. 2017).

Hand in hand with oxygen consumption, mitochondrial membrane potential flux is another measure of mitochondrial function. The mitochondrial membrane potential is created by the traffic of electrons from Complexes I, III, and IV into the intermembrane space. This inequity of protons in the outside of the mitochondrial matrix creates a proton gradient that provides the transmembrane potential needed to create ATP. Consequently, the changes in mitochondrial membrane potential are tied with the accumulation and later alleviation of the pressure produced by the H+ in the intermembrane space and the shuttling of electrons down the respiratory complexes. Several colorimetric methods have been developed to track mitochondrial membrane potential (Krumschnabel et al. 2014). Most commonly used is tetramethylrhodamine methyl ester (TMRM e.g., (Tehrani et al. 2019)), a molecule

small enough to be taken into the mitochondrial matrix during times of hyperpolarization and released upon membrane depolarization.

Previous clinical examination of large-scale orthopedic trauma has been postulated to increase sedentarism (Maggio et al. 2017), and subsequently the risk of metabolic comorbidities (Rynders et al. 2018), but measuring whole-body metabolic parameters in animal models has been historically challenging. Advancements in equipment have made it possible to overcome some of these challenges and were recently used to study VML-injured mice (Dalske et al. 2021). The equipment used is called a comprehensive lab animal monitoring system (CLAMS), which is a closed environmental and metabolic chamber that can control and measure temperature and ventilatory gases (O_2 and CO_2) used to calculate VO_2, respiratory exchange ratios (RER), and metabolic rate. The chamber also includes infrared beams in the X, Y, and Z planes that can track rest and movement patterns, which are used to determine physical activity. Other modular features include periodic body mass measurements, wheel/treadmill running, and food/fluid intake monitoring. The versatility of the metabolic chambers combined with robust monitoring of animal activity makes it an excellent tool for assessing animals after injury such as VML. In fact, the aforementioned study revealed that VML-injured mice maintain similar physical activity levels to uninjured controls (Dalske et al. 2021), which is supported by wheel running experiments (Southern et al. 2019), but is an unexpected finding based on clinical observations following traumatic injury (Ceroni et al. 2011). Despite similarities in physical activity, VML-injured mice demonstrated whole-body metabolic impairments, referred to as metabolic inflexibility (Kelley et al. 1999). Specifically, VML-injured mice were unable to effectively shift between carbohydrate and lipid utilization during active hours, suggesting that metabolic impairments previously observed at the cellular level extend beyond the relatively small injury site to negatively impact whole-body metabolism. Of course, additional studies using advanced whole-body metabolism and physical activity monitoring are needed to connect VML with chronic metabolic outcomes. On the other hand, equipment such as the CLAMS units could also be used in combination with regenerative and rehabilitative interventions following VML injury to see if physical activity and/or whole-body metabolism can be improved.

6.3.3 Loss of Regenerative Capacity

Successful restoration of injured muscle tissue requires the repair and/or regeneration of muscle fibers, which are highly organized in bundles with specific structural and contractile properties. Typically, skeletal muscle has a robust endogenous regenerative response following acute injuries (exercise-induced, strains, contusions), which follow a canonical regenerative order that ultimately restores tissue structure and function to, or even above, pre-injury levels (Fig. 6.2) (Wosczyna and Rando 2018). This regenerative process occurs over the course of several days to weeks in mammals and follows a time-dependent, energy-demanding sequence

characterized by robust infiltration and adaptation of immune cells (McLoughlin et al. 2003), expansion and migration of muscle progenitor cells (i.e., satellite cells, Fig. 6.2), and remodeling of the vasculature, motor units, and extracellular matrix (ECM) (Hyldahl et al. 2015). Unfortunately, VML injuries do not follow the same regenerative time course and ultimately fail to restore functional muscle tissue to its original form (Corona et al. 2013a).

A number of preclinical VML studies do show an initial increase in muscle function following VML injury, suggesting that the endogenous regenerative repair response may be present in the early phase of the injury (Aguilar et al. 2018; Greising et al. 2017; Corona et al. 2018a). Upon further inspection however, this response is characterized by irregular waves of inflammation, an absence of progenitor cell expansion and migration into the defect area, and excessive formation of ECM collagens that fill the VML defect area with scar tissue (Greising et al. 2017). Thus, the early improvements in function may not indicate regeneration, but instead an unregulated cellular response with a passive increase in force transmitting collagens that ultimately overwhelm the tissues endogenous regenerative capacity.

6.3.3.1 Experimental and/or Clinical Outcome Measurements

A significant portion of VML-related work is focused on developing treatments to restore order and function to the cellular response within the VML defect area. One of the primary techniques used to assess regenerative capacity is the histological identification of cells and their structures using various cellular identification techniques (e.g., flow cytometry) and imaging (e.g., microscopy, ultrasound, MRI).

For the quantification of mononuclear cell populations, the most promising method of interest would be to use flow or mass cytometry. These techniques use fluorescently labeled antibodies or unique metals, respectively, to identify cell populations with high efficiency. What makes this approach valuable is that it can accurately identify and differentiate between multiple cell populations in a given sample or whole muscle, drastically reducing the amount of time spent analyzing images. Pathological studies have shown extensive infiltration and persistence of immune cells into the VML injury using flow cytometry (Hurtgen et al. 2017; Hymel et al. 2021). If possible, more studies should consider using this technique to better understand the dynamic inflammatory response of VML injury following the use of regenerative and/or rehabilitative interventions. However, a specific limitation to this method with VML injury is that it lacks the temporo-spatial benefits found using microscopy. For example, histological samples reveal that Pax7-expressing satellite cells are found in the remaining muscle and border regions of the VML injury, yet they are rarely seen infiltrating the defect area (Greising et al. 2017). This type of spatial observation would go unnoticed using flow cytometry alone. Thus, the benefit of microscopy is that it provides temporo-spatial context to the dynamic response in cellular populations. Furthermore, microscopy can be used to assess other muscle fiber characteristics.

Quantifiable measures of muscle fiber regeneration are typically done using microscopy, and include the identification of newly forming muscle fibers, total muscle fiber number, the measurement of muscle fiber cross-sectional area, and in some cases the appearance of centralized nuclei. To differentiate between existing and newly forming muscle, various myosin heavy chain (MyHC) isoforms can be used to identify mature fibers, while embryonic MyHC is acutely expressed in regenerating fibers. If a regenerative cell therapy approach is used, such as stem cells or minced muscle graft transplants, investigators should consider using fluorescently tagged cells or tissue. Thus, the contribution of the cell therapy or tissue can be easily distinguished from the endogenous fibers. In otherwise healthy muscle, muscle function is highly correlated with muscle size and number, and the cross-sectional area of the muscle fibers is a good indication of the muscle's capacity to generate force and adapt. The extent to which these relationships are maintained in VML-injured muscle is unclear.

6.3.4 Loss of Functional ECM Replaced with Pathologic Fibrosis

The complex web-like structure that surrounds the muscle tissue is known as the extracellular matrix (ECM), which is an extension of the tendon, transmitting the contractile forces generated from muscle fiber contraction to the bone for movement. Beyond the ECM's contribution to movement, there is mounting evidence to support its critical role in the healing of injury muscle tissue (Gillies and Lieber 2011; Hyldahl et al. 2015). Skeletal muscle ECM is composed of various collagen isoforms, proteoglycans, and glycosaminoglycans that serve as a scaffolding to provide structural support for the muscle fibers, vasculature, nerves, and the diverse cellular populations that reside within the tissue micro-environment. Healthy muscle has a relatively consistent ratio of ECM in comparison to myofiber cross-sectional area. As muscle fibers hypertrophy, there is a corresponding increase and complex remodeling of ECM content (Mendias et al. 2017). However, muscle wasting conditions such as muscular dystrophies appear to disrupt this relationship, resulting in greater ECM accumulation with smaller muscle fibers. The unbalanced accumulation of ECM (pathologic fibrosis) results in deterioration of muscle quality and poor muscle function by reducing joint range of motion and maximal force production. Excessive ECM accumulation is one of the primary characteristics of VML injury (Corona et al. 2018b). Following the blunt removal of muscle fibers, mononuclear cells infiltrate the defect area and deposit thick layers of ECM that create a ball of scar tissue to replace the muscle fibers.

Following acute injuries (i.e., strains, or exercise induced) the ECM undergoes a remodeling phase that is vastly important for healthy regeneration and repair of the damaged myofibers. This transient remodeling phase is referred to as the transitional ECM, and includes the enzymatic breakdown and cellular upregulation of collagens

and adhesion-related proteoglycans (i.e., tenascin-c, fibronectin, hyaluronic acid) (Calve et al. 2010). The transitional ECM acts as a highway for cells to migrate toward the injury sight and facilitate the repair/regeneration process. The disappearance of a transitional matrix can lead to widespread fibrogenesis, as is observed in other skeletal muscle disorders (i.e., muscular dystrophy) and aging (Sorensen et al. 2018). There appears to be a tipping point in more severe pathologies, where muscle regeneration is repressed, and ECM deposition progresses uninhibited (Fig. 6.2). For example, the blunt removal of muscle fibers, resident mononuclear cells, and ECM components following VML injuries results in the aggressive formation of highly cross-linked or dense collagens (Hoffman et al. 2021). Indeed, the loss of functional ECM appears to limit the regenerative potential of the muscle, likely contributing to failed migration of satellite cells from the remaining tissue into the defect area. Notably, the frank removal of the existing ECM eliminates the structural support system that is needed for new muscle fibers to grow and survive. Therefore, regenerative medicine interventions have attempted to address these issues with an approach of inserting various biomimetic scaffolds into the VML defect area to establish a physical support system for angiogenesis, myogenesis, and reinnervation (Aurora et al. 2015; Haas et al. 2019). This approach has had varying levels of success, specifically in the ability to restore function. Going forward, it will be critical to find a balance between functional ECM remodeling and inhibition of fibrogenesis.

6.3.4.1 Experimental and/or Clinical Outcome Measurements

As work progresses to better understand and mitigate the accumulation of fibrosis following VML injury, there are several methods that can be used to evaluate ECM content in skeletal muscle. For clinical or repeated measures less invasive techniques are available; these include ultrasound or passive muscle function examinations (Corona et al. 2020). Modern advancements in technology, real-time imaging, and wide spread availability make ultrasound an excellent option for human studies. Briefly, images are collected utilizing B-mode and shearwave elastography as a mechanism to directly examine mechanical properties of skeletal muscle. This is done at various joint angles when an external force is applied to the tissue being imaged and the amount of displacement or "stiffness" is measured. Stiff tissue tends to deform less and show less strain compared to compliant tissue (Corona et al. 2020). To carry out passive muscle stiffness examinations, the limb of interest is secured to a dynamometer and passively rotated at the joint. Passive force measurements are collected at various joint angles where an increase in resistance for a given joint angle indicates greater muscle stiffness (Greising et al. 2018; Corona et al. 2020). Importantly, these measures can be examined longitudinally in humans to determine the efficacy of a given treatment or the progression of the VML pathology.

The assessment of ECM content at the cellular level can be performed using more invasive techniques that require the collection of muscle samples. A commonly used method is histological analysis of skeletal muscle cross sections. Notably, different

histological techniques are used to quantify ECM content; these include electron microscopy, Masson's trichrome stain, picrosirius red stain, and antibody labeling of specific ECM markers. Each approach uses a corresponding microscope to take full advantage of the muscle sample. Generally speaking, electron microscopy allows for high definition qualitative assessment of the ECM structure (Gillies et al. 2017; Gao et al. 2008; Järvinen et al. 2002; Purslow and Trotter 1994), while Masson's trichrome and picrosirius red quantitatively assess overall ECM content (Ramaswamy et al. 2011; Greising et al. 2017). With the addition of a polarizing filter, the picrosirius red stain can be further used to assess loosely vs. densely packed collagen (Hoffman et al. 2021; Dearth et al. 2016; Smith and Barton 2014; Arruda et al. 2007). Lastly, immunohistochemistry can be broadly used to label specific collagen isoforms and other ECM proteins (e.g., laminin, fibronectin, dystrophin) (Piñol-Jurado et al. 2018; Cáceres et al. 2000; Zanotti et al. 2005) or ECM-related growth factors (e.g., TGF-β or connective tissue growth factor) (Li et al. 2004). Histological quantification of ECM content is typically expressed as a measure of area.

Total collagen, collagen isoforms, and fibrotic markers can also be examined at the protein and gene level. After muscle samples are properly homogenized and processed, biochemical assessment of total collagen can be done using a hydroxyproline assay. Furthermore, antibody-based probing (e.g., ELISA) can be used to examine specific collagen isoforms and proteins of interest. Lastly, some proteins, such as TGF-β, may also be measured using multiplexing assays, which have the distinct advantage of including a host of markers in a single sample. Notably, recent advancements in proteomics and transcriptomics reveal that only a portion of the gene expression is revealed in the protein content, suggesting that protein measurements more accurately reflect the cellular response. Nonetheless, gene arrays, qRT-PCR, and next-generation sequencing analysis are widely used research techniques that provide additional insight (Aguilar et al. 2018; Corona et al. 2017b; Greising et al. 2017; Greising et al. 2018; Cherry et al. 2021; Nuutila et al. 2017; Aurora et al. 2014). The advantage of gene arrays is that a large number of genes can be tested to identify enriched pathways of differentially expressed genes that could serve as potential therapeutic targets. The advantage of sequencing is increased flexibility with hardware design and the probability of discovering unknown gene variants. Overall, the extracellular matrix is a critical and often overlooked component of skeletal muscle health. As more treatments for VML are developed and tested, the fibrotic response will continue to be an area of interest for achieving success.

6.3.5 Extensive and Irregular Inflammation

The extent of damage caused by VML injuries is typically beyond the muscle's natural ability to regenerate. Regeneration takes place under complex inflammatory conditions (Grounds 2014), entailing the activation of the innate immune system and

recruitment of effector immune cells (Fig. 6.2). Early stages of healing involve a pro-inflammatory environment, where resident macrophages are polarized toward the M1 phenotype and, along with other recruited immune cells, facilitate the repair process by breaking down cellular debris. This process is also important for the activation and migration of satellite cells to the area of injury. Under normal conditions, this pro-inflammatory milieu subsides as damaged cells and extracellular matrices are cleared away. Macrophages will then polarize toward the M2 or anti-inflammatory phenotype which will stimulate satellite cell differentiation and myofiber repair.

VML-injured muscle maintains an increased expression of chemotactic, inflammatory, and immune cell infiltration gene transcripts (Greising et al. 2016, 2017). Where other muscle injuries see a decrease in inflammatory signaling a few days after injury, VML has been shown to maintain this sustained expression even 28 days following injury (Aguilar et al. 2018). Similar to other models of severe muscle trauma, VML presents with an inflammatory profile, producing an abundance of COX1/2, CSF2, IL6, MIF, and STAT3. Moreover, immune pathways, such as complement activation, and fibrosis-inducing pathways, like TGF-β1 and Wnt, remain constantly activated after injury. This is important because TGF-β1 and Wnt control the behavior of multipotent fibro-adipogenic precursors living in the cellular niche of the muscle, which are thought to be responsible for the increased fibrosis in the VML defect area and the suppression of satellite cell activation, respectively.

Increased fibrosis in the muscle defect creates a myogenesis-inhibitive feedback loop, as fibrotic tissue secretes cytokines that block myogenic differentiation factors such as MyoD, MyoG, and Mef2. Fibrosis and prolonged immune cell infiltration and action create an unfavorable environment where remodeling enzymes are constantly activated to clear away ECM that is interfering with the growth of new myofibers, leading to further inflammation (Shayan and Huang 2020). This environment is unreceptive to therapies and causes a degenerative phenotype in satellite, immune, and fibro-adipogenic precursor cells (Larouche et al. 2018). Therefore, therapeutic candidates must be able to immunomodulate the cell niche to usher in the anti-inflammatory stages of repair, when appropriate, stave off fibrosis and adipogenesis, preserve the remaining muscle, and positively influence satellite cells.

6.3.5.1 Experimental and/or Clinical Outcome Measurements

Inflammation is a necessary component of wound and tissue healing (Greising et al. 2020). High temperature, increased fluid retention, and the recruitment of effector immune cells are all part of a highly coordinated effort to clear away debris and allow for the regeneration and remodeling period to commence. However, as it was established earlier, the inflammatory period during VML injuries tends to subsist further than what is necessary or beneficial for the tissue. This extended inflammation is thought to hinder the regeneration process by breaking down newly formed tissue and causing damage to any growth factors, cells, or adjuvants delivered to the area of injury.

Monitoring immune cell infiltration or the product of immune cell action in skeletal muscle has been previously explored in models of VML. Gene expression for protein products and proteases secreted by immune cells has been previously used to show inflammatory response in VML (Corona et al. 2013a; Hurtgen et al. 2016). Masson's trichrome and hematoxylin and eosin staining have been used to show the effects of immune cell infiltration in injured muscle. Recent reports have also used fluorescence activated cell sorting (FACS) to identify immune cell populations in a longitudinal assessment of VML injuries (Hymel et al. 2021). Additionally, the availability of ELISA and magnetic multiplex assays allows for the testing of a wide array of immune secreted factors, specifically cytokines such as IL-1β and IL-6.

6.3.6 Loss of Innervation

A motor unit is made up of a motor neuron and the skeletal muscle fibers innervated by that motor neuron's axonal terminals, i.e., individual neuromuscular junctions (NMJs) on each fiber. The NMJ is a specialized chemical synapse that plays a critical role in muscle contraction where an action potential passes from the motor neuron to the individual muscle fiber to convert chemical energy into mechanical energy via the cross-bridging of actin and myosin. Due to the critical nature of the NMJ in facilitating muscle contractions, it has been shown to be a point of failure in aging, disease, and skeletal muscle injury models such as denervation injury or tissue ischemia. Indeed, the intimate physical connection between the nerve and muscle is critical for the function, survival, and morphology of the muscle fibers. Conversely, the loss of innervation is a major contributor to the pathological loss of muscle fibers, mass, and strength.

Until recently, the pathology of innervation following VML injuries had not been thoroughly examined. It was generally assumed that a significant number of NMJs were removed or damaged in conjunction with the loss of other structural and functional properties within the muscle tissue. However, evidence to support this claim is limited and can vary depending on the injury model and sequela of the injury. In one study specifically, acetylcholine receptors were used as a marker to count NMJs throughout the muscle, revealing significantly less clusters following a VML injury when compared to the uninjured contralateral limb (Anderson et al. 2019). This data supports the idea that NMJs are removed as a result of the injury model used in this particular study. However, it is important to note that the loss of innervation due to the direct removal of tissue may vary based on the size and location of the VML injury. Furthermore, the labeling of acetylcholine receptors alone, while beneficial to determine the number of NMJs, fails to account for other critical structures important for characterizing innervation. To this end, a recent study found progressive secondary loss of innervation up to seven weeks post-VML injury in the remaining tissue (Sorensen et al. 2021). This was done by examining the colocalization of pre- and postsynaptic NMJ structures. These findings are

supported by a previous study which showed significant axotomy following VML injury (Corona et al. 2018a), suggesting that the impact of VML extends well beyond the initial injured area (Fig. 6.2). This may help explain the long-term dysfunction that persists following VML injures as well as highlight innervation as a promising therapeutic target for improved healing and restoration of function in future studies.

6.3.6.1 Experimental and/or Clinical Outcome Measurements

As muscle and nerve communicate in a bidirectional way, being able to measure motor unit defects separately from muscle defects provides insights into their pathophysiologic interplay. Therefore, considerations for future studies should understand that there are three main approaches used for identifying denervated muscle fibers. The first method is an indirect approach that combines electrical stimulation: direct muscle membrane simulation and stimulation through the nerve to test neuromuscular transmission failure (Greising et al. 2015). The second method also measures innervation indirectly using a continuous neural stimulation protocol to deplete innervated muscle fibers of glycogen, followed by histological identification of myofiber glycogen content (Corona et al. 2013a). The third method is performed by directly examining the integrity of the NMJ using immunohistological probes that identify NMJ structures. In the first method, the comparison of the muscle contractile response elicited by nerve stimulation and the response of the same muscle evoked by direct stimulation of its membrane has been proposed as an indirect measurement of NMJ functionality. Indeed, since membrane stimulation bypasses neurotransmission signaling, any differences in the two contractile responses may be ascribed to changes in the NMJ. This approach has been extensively used in porcine, rat, and mouse models of aging and disease. For the second method, the muscle undergoes an intense contractile protocol involving several bouts of electrically stimulated muscle contractions. Once the bouts are completed, the tissue is immediately harvested and probed for identification of glycogen stores. Innervated fibers will be void of glycogen, while denervated fibers will maintain glycogen stores (Corona et al. 2013a). For the third method, antibodies are used to label the presynaptic terminal of the NMJ along with the motor axons. Additionally, a neurotoxic venom from the Taiwanese many-banded krait, known as α-bungarotoxin, is used to label the postsynaptic terminal. When used in combination, investigators can determine the innervation status of the NMJ. Complete colocalization of the pre- and postsynaptic terminals indicates fully innervated, while the partial or complete absence of the presynaptic terminal would represent denervation. Furthermore, morphological characteristics such as fragmentation, poly-innervation, axon sprouting, and axon thinning, which are thought to play a role in NMJ remodeling during development or following acute injury, can be identified. When using this approach, it is highly advised to consider the anatomical structure and location of the NMJs. Indeed, the pre- and postsynaptic terminals of the NMJ and corresponding axons run longitudinally along the muscle fibers, indicating that thickly cut sections or whole tissue samples be used in the longitudinal

orientation. It is possible to use the typical cross-sectional orientation commonly seen with skeletal muscle; however, a cross-sectional orientation may fail to identify important data concerning the NMJ.

It is important to note that a number of studies have identified newly forming NMJ structures in and around the VML defect area following the use of regenerative interventions (Turner et al. 2010; VanDusen et al. 2014), suggesting that the potential to reinnervate fibers within the defect area is possible. Additionally, the discovery that NMJ size in the remaining tissue is preserved (Sorensen et al. 2021) coupled with lack of motor unit death in the spinal cord (Corona et al. 2018a) is further confirmation that innervation can likely be rescued. As such, future studies need to strongly consider targeting and performing in-depth analysis of innervation following regenerative and rehabilitative interventions, as this may be an undervalued explanation for regenerative and functional deficits.

6.3.7 Vasculature

The vascular network within skeletal muscle is highly organized into branching networks of smaller arteries, arterioles, capillaries, and venules making up the microcirculation. Microcirculation is critical for the exchange of water, gases (e.g., O_2, CO_2), nutrients (e.g., glucose, free-fatty acids), and waste products between the muscle tissue and blood. Skeletal muscle metabolic demand can increase several hundred-fold with incremental levels of physical activity, meaning perfusion and diffusion properties of microcirculation are important variables for sustained muscle function. There are three factors influencing the severity of VML injury on muscle vascularity: (1) the amount of microcirculation lost with the physical removal of the muscle, (2) disrupted microcirculation perfusion and diffusion in the remaining muscle, and (3) the extent to which re-vascularization can occur in the remaining muscle and/or tissue that replaces the muscle lost during the VML injury (Fig. 6.2). There is evidence of vascular growth near the injury site in the weeks following VML injury in rodent models, but the extent of perfusion to those and existing networks remains questionable (Anderson et al. 2019; Southern et al. 2019; Li et al. 2017). Ultimately, the loss of microcirculation after a VML injury has been implicated in contributing to mitochondrial dysfunction, impaired muscle stem cell activation, and extensive fibrosis in the remaining tissue. The microcirculation is a critical target for regenerative medicine and rehabilitation approaches alike to improve the overall quality of muscle post-VML injury.

6.3.7.1 Experimental and/or Clinical Outcome Measurements

There exist several outcome measurements for vascularization of both the remaining muscle and defect area post-VML injury that vary in accounting for the quantity of vessels and function of those vessels. Capillary density and the capillary-to-fiber

ratio can be assessed histologically with alkaline phosphate or capillary-specific antibodies (e.g., CD34) and skeletal muscle fiber membranes (e.g., dystrophin) (Dalske et al. 2021; Machingal et al. 2011). Capillary density and capillary-to-fiber ratio can indicate the theoretical perfusion and diffusion limitations within muscle, i.e., fewer capillaries increase the diffusion distance from any point within a muscle fiber to the nearest capillary. Greater diffusion distances negatively affect the ability of muscle fibers to exchange nutrients, gases, and waste products. In normal physiological settings, only 40–60% of capillaries will be perfused with blood at any time; therefore, greater physiological relevance is placed on outcome measurements of perfusion. Contrast imaging in which a radioactive dye is pushed through the left ventricle into circulation and imaged using μCT can provide a closer representation of vascular beds and their perfusion parameters (Southern et al. 2019). There are many examples of this approach being used to interrogate the pathophysiology of VML injuries as well as monitor vascularization of tissue transplants into the defect area left by a VML injury. Laser Doppler flowmetry is a noninvasive technique that relies on the Doppler shift of laser light to detect blood flow to skeletal muscle. Another noninvasive technique is near-infrared spectroscopy that measures changes in near-infrared light absorption that can be inferred as blood flow and oxy/deoxyhemoglobin levels. There are some examples of these noninvasive techniques being used to validate regenerative medicine approaches following VML injury, but in general noninvasive blood flow imaging studies are a challenge area for advancement of understanding the VML pathology.

6.3.8 Long-Term Disability and/or Delayed Amputation

The path of VML-injured patients to even modest improvements in functional capacity is currently long and hindered by comorbidities. Rehabilitation alone is unlikely to attenuate the sequela of pathology in the remaining muscle, leading to chronic limb dysfunction. A study of medically retired battlefield-injured servicemembers reported that a muscle condition contributed to 53% of the total disability rating, which reflects a servicemember's physical fitness and disability payment (Corona et al. 2015). Additional rehabilitation time does not significantly improve physical fitness and further deterioration has been reported (Rivera and Corona 2016), at which point late-stage amputation becomes a primary concern (Krueger et al. 2012; Stinner 2016; Stinner et al. 2010). Functional deficits secondary to VML injuries likely contribute to late-stage amputation. In theory, a proportion of late-stage amputations could be avoided if clinically viable treatment approaches were available to improve the regenerative potential of the remaining muscle. For the VML-injured patient, any functional gains beyond those achieved with rehabilitation are expected to have a considerable impact on quality of life and as such regenerative rehabilitation may be an ideal tool to accomplish this goal.

6.4 Rehabilitation Approaches

6.4.1 Model Limitations

Animal models are invaluable tools for disease research. They allow for the profound study of biochemical pathways and physical adaptations in a living system, and provide a platform for the screening, testing, and developing of new pharmaceuticals. Despite their usefulness, there are many instances in which disease progression or treatment response between animal models and humans generally differs (Hartung 2008; Hackam 2007; Hackam and Redelmeier 2006). For example, animal VML injury models are highly controlled and may not always translate to what is observed in clinics based on injury location, size, species, age, sex, and severity. Not to mention the anatomical and ambulatory differences between animals and humans. While these systems serve to fill a technical gap, there are disadvantages and limitations that must be kept in mind when inferring or translating study results to a human context.

6.4.1.1 Physical Constraints

A large number of orthopedic models are developed in rodents or large animals, like sheep or pigs (Hartung 2008; Moran et al. 2016; Rhrissorrakrai et al. 2015). The similarity in muscle composition offers valuable insight on the biochemical pathways activated during injury and recovery and serve as an excellent testbed to gauge biological response to therapies. However, data cannot be directly correlated to human condition. As quadrupeds, weight distribution, pennation and torque angles, and muscle position vary considerably to that of humans (Hartung 2008; Moran et al. 2016; Shayan and Huang 2020). Therefore, the strain placed on a muscle in an injury model may not accurately portray the actual injury.

The magnitude and location of VML injuries result in considerable heterogeneity and necessitate a cadre of therapies (Rodriguez et al. 2020). For instance, a small VML defect isolated to a peripheral portion of a muscle unit may be best compensated by synergist hypertrophy mediated through physical therapy, whereas VML defects have been shown to not respond to conventional physical therapy and may require regenerative medicine augmentation. This could be due to the heterogeneity of injury defect size and locations seen in clinic, which make the standardization of treatment protocols difficult. Regenerative treatments for VML have utilized various strategies but require significant further advancement to be of therapeutic benefit to patients presenting with acute or chronic VML injury.

VML injuries are known for having long periods of recovery during which the muscle undergoes several bouts of inflammation and the limb remains mostly immobile before healing due to commonly concomitant bone injury (Corona et al. 2015). By virtue of the animals these models are developed in, recovery from VML occurs in a significantly shorter time frame than their human counterparts. This is

especially true in rodents, where injured subjects are able to ambulate, run, and even jump within minutes of recovering from anesthesia. Moreover, there is no period of limb immobilization or disuse unless the animal is physically restrained or the nerve innervating the muscle group of interest is severed. While it is advantageous for researchers to have a reduced window for disease progression and healing, it can sometimes lead to treatments being suspended during clinical trials despite the fact that the preclinical data were promising.

6.4.1.2 Metabolic Constraints

A major pitfall that hinders models of nearly every disease is the determination of appropriate dosages for pharmacological adjuvants (Hartung 2008). In some cases, there are metabolic redundancies in animals not present in humans that affect the organism's response to a treatment (i.e., immune system response and robustness being distinctly different) (McGonigle and Ruggeri 2014) that leads to a discrepancy in the predicted bioavailability in humans (Musther et al. 2014). Though there are mathematical models that can help predict dosing for humans, there are still major lags in first-in-human (FIH) estimations (Zou et al. 2012) to achieve the observed effect from animal models. This is especially important when working on new dosages for off-label indications of medications already on the market.

6.4.1.3 Functional Assessments and Outcomes

In clinical settings, patients who have suffered VML injuries are evaluated on a wide range of parameters, including clinical photography and gait analysis, range of motion, muscle strength, and isometric muscle function (Sicari et al. 2014). All these evaluations are directed at assessing the muscle's performance following injury and subsequent recovery. Assessment and outcome measures are often guided by the longtime goal of the research project and are not a standard or comprehensive evaluation of muscle soundness. Therapies that aim to address filling the defect area left behind after VML injuries may not wish to include the same outcome measures as those therapies intending to prime and improve the remaining muscle tissue. As an example, a number of VML studies use histological evaluation (e.g., fiber number, fiber diameter, MyHC expression) as the metric for muscle regeneration in the defect area but fail to include measures of strength. This type of outcome not only disregards the importance of proper muscle function as the desired result, but it wrongly equates hypertrophy with functional improvement. As such, standardization of outcomes measures to match those seen in clinical settings is a necessary addition to the VML model field. In order to maximize the ability of VML studies to inform future evidence-based approaches, we propose an outcome measurements hierarchy to consult during study design (Fig. 6.3). This model prioritizes functional outcomes with clinical comparisons, e.g., gait analysis and muscle strength, and gives less priority to measurements that cannot provide direct interpretation on the

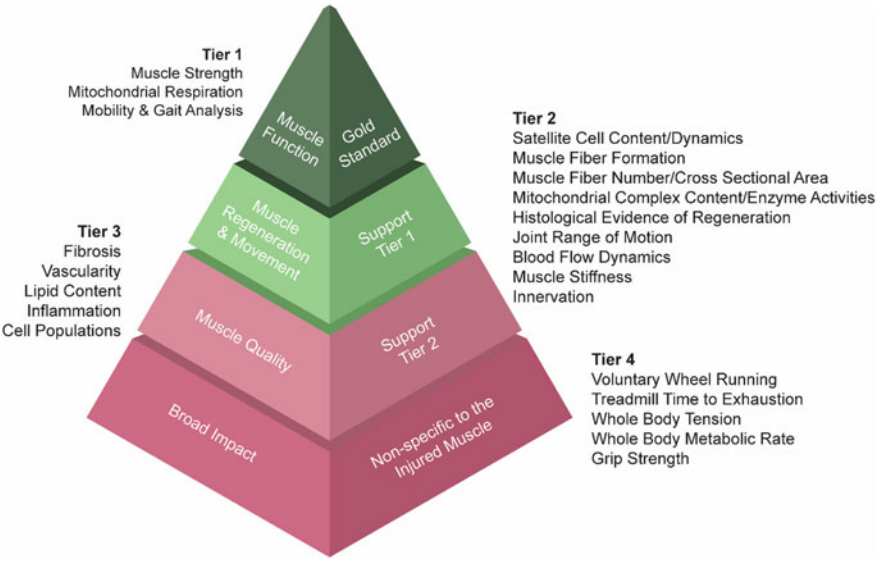

Fig. 6.3 A four-tier model for evaluating outcome measurements for VML. Tier 1 includes outcomes with clinical similarities; Tier 2 are outcomes that most directly influence Tier 1; Tier 3 provide broad evidence of muscle quality; and Tier 4 are outcomes involving other systems in the body making direct interpretation of muscle function difficult

health of skeletal muscle; e.g., treadmill running involves the cardiovascular and respiratory systems.

Models are not meant to be perfect representations. They exist to minimize the variables within a system to isolate a few tangible elements. Acknowledging the limitations within that system increases the study's robustness and prevents overselling of positive results. Moreover, it is each researcher's responsibility to create model systems that can be accurately translated to human research and that add value or improve upon the current understanding of VML pathophysiology.

6.4.2 Physical Rehabilitation

Currently, no corrective physical rehabilitation guidelines exist for the treatment of VML injuries. Perhaps, it is due to the uncertain adaptability of the remaining skeletal muscle using rehabilitation techniques. Various models of physical rehabilitation following VML have been used in preclinical models, including voluntary wheel running, forced treadmill running, chronic-intermittent electrical nerve stimulation to induce concentric or eccentric contractions, and/or passive range of motion exercises (Washington et al. 2021; Quarta et al. 2017; Southern et al. 2019; Greising et al. 2018; Aurora et al. 2015). Collectively, these diverse approaches have shown that modest contractile adaptations are possible. Meanwhile, clinical reports have

shown that VML patients see moderate improvements before their progression halts and further rehabilitation, regardless of the type or intensity, fails to improve function. Collectively, rehabilitation following VML has resulted in modest, if any, improvement in muscle function without any physiological rationale or mechanistic understanding for the lack of significant response.

Despite the discouragement of unsuccessful attempts to improve functional outcomes following VML injuries, it should not be interpreted to suggest that all forms of physical rehabilitation will fail. It is merely that the appropriate combination of rehabilitative and regenerative medicine therapies has not been identified. Furthermore, a rehabilitative standard of care does not exist and the variables that need to be considered for are extensive, including the type, timing, volume, and intensity of the therapy. Future research needs to focus on the synergistic potential of regenerative rehabilitation, where regenerative therapies are combined with rehabilitation in an attempt to restore functional muscle tissue to VML-injured patience.

6.5 Regenerative Medicine

6.5.1 Cell Therapies

Skeletal muscle stem cells, known as satellite cells, have a unique ability to fuse to existing fibers (hypertrophy) or fuse together to form new fibers (hyperplasia), making them indispensable for the endogenous repair and regeneration of skeletal muscles. Satellite cells reside underneath the basal lamina on skeletal muscle fibers, typically in a quiescent state. Following injury, satellite cells become activated and migrate to the injury site where they expand and a portion of the cells differentiate into functional muscle tissue to repair, regenerate, and restore the muscle to its previous healthy state. The remaining portion of satellite cells returns to their original quiescent state to preserve the stem cell pool. Unfortunately, the ramifications of VML on satellite cells within the remaining muscle are not completely understood and current strategies aimed at exploiting satellite cells as a source of regenerative medicine have been hampered with challenges. For instance, across various VML injury models and species, satellite cells demonstrate limited migration following injury. Specifically, all satellite cells are found within the remaining tissue, while the injury area is devoid of a satellite cell presence or meaningful regenerating muscle fibers. Studies have shown that gene expression of Pax7 (a satellite cell marker) initially increases post-VML, but its expression declines chronically, suggesting a lack of endogenous proliferative capacity (Aguilar et al. 2018). In an effort to overcome these challenges, researchers have attempted to add various donor cells (e.g., satellite cells, inducible pluripotent stem cells, mesenchymal cells) into the VML defect area (Baker et al. 2017; Merritt et al. 2010; Mori et al. 2015; Oshima et al. 2014; Passipieri et al. 2017; Qiu et al. 2018; Quarta et al. 2017; Shi et al. 2009). A variety of approaches have been used to accomplish this, the most common being the application of purified satellite cells alone or in combination with hydrogels or

scaffolds. These approaches have shown varying results with limited impact on restoring muscle function. Some of the challenges with this approach have been linked to poor satellite cell engraftment. In other words, the cells fail to adhere to the existing tissue and have been described as having lost their "stemness." A possible explanation may be that in order to generate a sufficiently large pool of satellite cells, they must first undergo in vitro expansion. While this technique can quickly expand the satellite cell pool, it also depletes the cells of their unique stem cell characteristics. Therefore, another approach which has shown some promise for improving muscle function is the use of a minced muscle graft (Corona et al. 2013a; Ward et al. 2015), which replaces all endogenous cell types and the components of the ECM. This approach does not require the in vitro expansion of the satellite cells. Additionally, it maintains the cell's natural environment by delivering the ECM and basal lamina wherein the satellite cells typically reside. Moreover, resident cells such as fibro/adipogenic progenitors, which have been shown to promote satellite cell expansion, are readily present in close proximity to the satellite cell population. However, all of these techniques still present challenging obstacles that need to be overcome, especially when considering a translational approach from animal models to humans.

6.5.2 Pharmaceutical Adjuvants

As discussed throughout the course of this chapter, a common consequence of VML injury is the overwhelming accumulation of inflammation and fibrosis that impede the endogenous regenerative response of the remaining tissue. A possible solution to restore a portion of the tissue's regenerative potential has come through the repurposing of FDA-approved pharmaceutical agents that have shown beneficial effects in other disease or injury conditions. To this end, some studies have administered anti-fibrotic agents as a primary treatment source for VML, while others have used anti-inflammatories as a mechanism to mitigate tissue donor rejection or dampen the inflammatory response when implanting a foreign material (i.e., scaffold). In fact, few studies have examined the functional impact of anti-inflammatories as a single treatment approach. What has generally been found is that functional outcomes are no different when using an anti-inflammatory versus no use, while modest improvements in muscle function are observed when combined with a muscle graft treatment (Corona et al. 2017b; Goldman et al. 2020, 2021), indicating that a small synergistic effect may occur when anti-inflammatories are used in combination with other regenerative therapies.

To date, some anti-fibrotic treatments for VML have targeted various signaling pathways such as TGF-β (Garg et al. 2014a), FGF, PDGFRα/β, and VEGF (Corona et al. 2020), which have known roles in fibrosis development. Paradoxically, many of these pathways also support other cellular functions within the muscle tissue. Perhaps, it is not surprising then that the administration of an anti-fibrotic treatment can successfully prevent fibrosis. However, the anti-fibrotic treatments do not

typically address skeletal muscle contractile function and in some cases lead to further decline in contractile force (Corona et al. 2020; Garg et al. 2014a). This supports the idea that a portion of the early improvements in muscle function are likely driven by the accumulation of force transmitting connective tissue. Despite these findings, future studies should continue to identify potential targets for preventing fibrosis or combine the currently available anti-fibrotic treatments with other regenerative and rehabilitative therapies.

6.5.3 Growth Factors

Skeletal muscles' robust capacity to adapt is dependent on the type of stress that the muscle is exposed to. For example, the ablation of the gastrocnemius muscle will result in a compensatory hypertrophic response of the remaining plantaris muscle in rodents (Degens et al. 1995), attributed to the increased demand placed on the muscle. However, the ability to adapt to stress following VML injury is lost, such that compensatory hypertrophy of the remaining muscle does not occur. It is well known that certain signaling pathways are activated as a result of injury or exercise to stimulate adaptation. The molecules that drive these adaptations are proteins commonly referred to as growth factors. It is thought that by manipulating endogenous signaling pathways following VML injury, it may be possible to rescue the adaptive potential of the remaining tissue. As a result, growth factors have been used alone or in combination with engineered muscle constructs to deliver a sustained concentration of the desired molecule during the recovery process, while also providing additional structural support to injured muscle. The results have presented both promising and conflicting outcomes, which are likely dependent on the specific molecule used. For example, a VML injury treated with a keratin construct and basic fibroblast growth factor (bFGF) showed poor force output two months after implantation (Baker et al. 2017). This may be partially explained by the discovery that molecules such as bFGF, hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) are already abundantly secreted into the VML defect area. However, when the keratin and bFGF construct was further supported by IGF, there was a synergistic increase in force that was not observed by either factor alone (Baker et al. 2017). On the other hand, molecules that are downregulated following VML injury may hold greater promise for rescuing a portion of the injured muscles' adaptive capacity. One such molecule is PGC-1α, when overexpressed in VML-injured muscle there was improved oxidative capacity (Southern et al. 2019). Overall, endogenous molecules do have the potential to improve VML outcomes if the molecule is suppressed following VML injury and used in combination with other therapies.

6.5.4 *Biomaterials*

Despite the fact that the term "biomaterial" has only recently come into use, humans have been using materials to aid in healing for centuries (Ratner and Zhang 2020; Migonney 2014) as noted in Chap. 1. In early civilizations people used wood, bones, animal teeth, and sinew for dentistry applications, making prosthesis for amputated or withered limbs, and sutures. As technology evolved, so did practices. The field reached a new cusp with the scientific breakthrough of polymer synthesis. Technology has allowed for these new implants to achieve better performance, functionality, and reproducibility than the naturally derived materials of old. Nowadays, the term biomaterial is used as a broad category that includes medical implants, aids for healing and regenerating human tissue, molecular probes, biosensors, and drug delivery systems.

The study of VML injuries has brought with it a vast array of natural and synthetic polymer materials. These materials have been manipulated in a variety of shapes, including natural or native structure sponges, gelloids, and fibers. With the use of these scaffolds, researchers have sought to carry growth factors, cells, and pharmaceutical adjuvants to the injury defect in hopes of restoring muscle mass and function. More importantly, the use of biomaterials can open a new field of VML treatment, where the muscle environment can be modulated through chemical and spatial cues, priming the muscle for future physical therapy.

6.5.4.1 Biocompatibility and Biomaterials Presentation

With the increased availability of both natural and synthetic materials to aid in healing, came an interest in studying the interaction of materials with the environment in which they were to be introduced. Many early biomaterials were chosen on the basis that they remain "inert" or "non-fouling" within the body, not triggering a significant immune response or interacting with the tissue in any meaningful way (Ratner 2019). As the field has evolved, however, more importance is being put upon the materials being able to modulate the cell environment to promote healing rather than remain an inert substance (Abdulghani and Mitchell 2019). This brought about the new concept of biocompatibility–material-host synergy.

Biomaterials engineering is a highly interdisciplinary field that is under constant improvement. New synthesis and functionalization techniques allow for the modification of both naturally and synthetically derived biomaterials to fit nearly any need. Materials developed for implantable devices are tested for reactivity, as they typically go through the entire process of FDA approval, and their function may depend on their non-interaction with the organismal environment. Soft materials, non-devices such as beads, micro- and nanoparticles, gels, and scaffolds, inherently become part of the environment upon implantation. Their interaction with the host's environment is sometimes neglected, leading to failure in vivo despite its promise

in vitro. This issue may stem from the lack of knowledge of the cellular and molecular mechanisms activated by implantation leading to poor material stability.

The extracellular environment following VML injury is flooded with cell debris, signaling molecules, and immune cells simultaneously coordinating several complicated molecular mechanisms. Many of these mechanisms are tissue-specific and require different biochemical signaling pathways, macromolecules, and signal transducers. Both natural and synthetic materials can be modified or functionalized so their surfaces can positively influence the extracellular environment. Some of these modifications can include creating hydrophilic or hydrophobic interaction sites, attaching charged or protein-specific binding sites, loading the material with a payload that influences the muscle environment, and modifying degradation kinetics to fit the time frame desired for study. Additionally, the chemical properties of the base material, including the products of its degradation, can be used to the researcher's advantage to modulate cellular response.

Properties such as topographical features, stiffness, pore size, and cell binding sites will influence the effectiveness of the biomaterial (Rahmati et al. 2020). Many researchers have reported on the influence of physical characteristics of a biomaterial on the cell fate and survival of different cell types (Abdulghani and Mitchell 2019). The surface topography of a scaffold can affect the determination, adhesion, differentiation, and migration of resident stem cells, leading to unwanted cell fates, such as adipose or fibrotic tissue. The stiffness of the muscle determines the amount of torque that can be produced from voluntary contraction around an axis (Abdulghani and Mitchell 2019). For example, an overly stiff material (Young's modulus) deposited into the muscle defect of a VML injury could hinder contraction of the muscle as the tissue heals or not allow for the migration, binding, and proliferation of stem or satellite cells within the material pores. Conversely, materials pre-loaded with myoblasts or satellite cells with unfavorable physical properties may not be able to maintain cell viability in vivo or allow for the migration of those cells into the existing myofibers.

Thus, an effective biomaterial should not hinder but enhance the natural process of tissue healing. Researchers must consider the possibility that in vitro data collected on these materials may not represent the conditions present in the extracellular niche in vivo.

6.5.4.2 Natural Biomaterials

For centuries, humans have been using naturally based materials to help heal their maladies. Wood and bone were some of the first materials used to mend broken limbs and replace teeth. Currently, naturally derived materials are used as biomaterials for their availability, biocompatibility, and biomimetic properties. These materials can be divided into four broad categories: proteins, polysaccharides, glycosaminoglycans, and cellular matrices (Brovold et al. 2018). The field of VML research has seen representatives from all of these categories as vehicles for the delivery of cells and therapeutics into the injury defect. Figure 6.4 shows

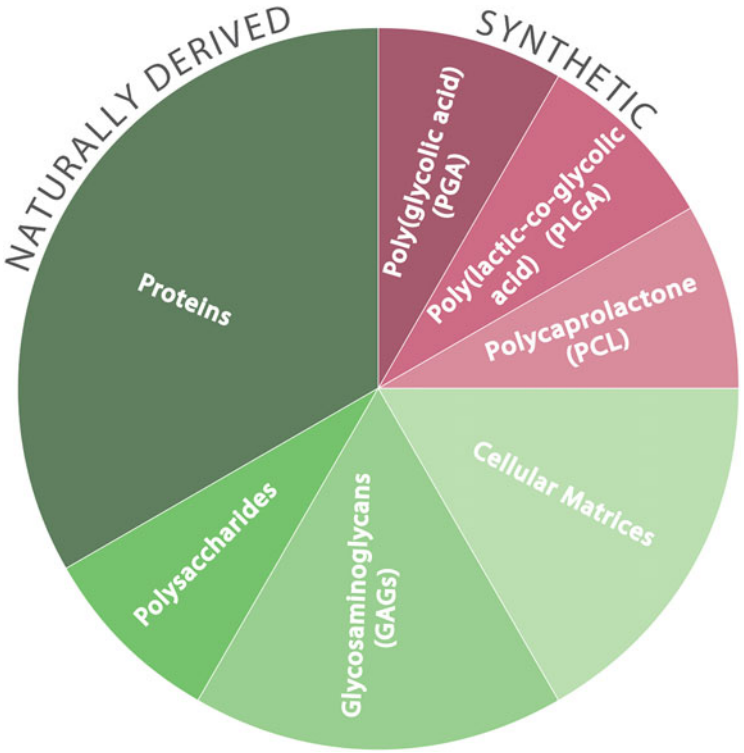

Fig. 6.4 Summary of materials tested for the treatment of volumetric muscle less injuries. The field of VML has seen a moderate number of studies where biomaterials have been engineered to either deliver a payload or encourage the growth of new tissue in the injury defect. Size and proportions of each category on the chart correlate with the prevalence for each material in research. Naturally derived materials make up the bulk of these studies, with protein scaffolds being the most popular. The use of synthetic materials has been significantly less explored, but it can be expected to increase as the field of bioengineering in VML injuries expands

different categories of scaffold materials that have been used in VML injury studies. The relative size of each category relates to the ubiquity of the material's use in research.

6.5.4.2.1 Protein Scaffolds

Collagen

Collagen is the most abundant structural protein in the body. It is secreted by fibroblasts as part of the ECM and provides support to diverse tissues, such as bones, tendons, and skin (Brovold et al. 2018). Though typically used as a scaffold for bone repair for its enhanced strength and mechanical properties, collagen can be modified to serve soft-tissue purposes. This material can be processed into beads,

sponges, meshes, and fibers that are conducive to cell growth, biocompatible, and biodegradable. It is easy to isolate and has many varieties, and its physical, structural, and chemical characteristics are well known.

Collagen is a popular material base for the construction of gels, fibers, meshes, and sponges used in VML injuries. Use of collagen sponges to treat VML muscle defects in the vastus lateralis muscle of rabbits resulted in regenerating myofibers on the periphery of the defect at 4 weeks and the partial connection of regenerating myofiber stumps at 24 weeks (Kin et al. 2007). Additionally, collagen scaffolds loaded with insulin-like growth factor-1 (IGF-1) have promoted proliferation and initiation of myogenic fusion of myoblasts, in vitro (Alcazar et al. 2020). Moreover, these biomaterials aided in the activation of satellite cells to the VML injury and scaffold site, in vivo.

Gelatin

Gelatin is a material derived from the hydrolysis of collagen (Brovold et al. 2018). Due to this, its structure and physical and chemical attributes are well known. It has an advantage over collagen in that it is readily soluble in aqueous solution, is commercially available, and can be easily cross-linked or chemically modified to fit a wide variety of applications. In a rat VML model, cross-linked gelatin hydrogels were used to deliver osteoactivin. Implantation resulted in immune cell encapsulation at 2 weeks, followed by cell infiltration into the gel at 4 weeks (Ma et al. 2017).

Gelatin has also been used in combination with other materials to create less expensive, stable, biomimetic sponges. The use of these sponges and a combination of gelatin, its polymer, collagen, and laminin have been shown to support C2C12 infiltration and survival in culture, as well as stimulate the expression of myogenic markers such as MyoD and MyoG. When implanted into VML defects, these sponges support satellite, endothelial, and inflammatory cell growth but did not offer significant myofiber regeneration after 2 weeks of treatment (Haas et al. 2019).

Keratin

Keratin encompasses a group of insoluble proteins that associate as intermediate filaments (Rouse and Van Dyke 2010). These proteins are broadly divided into two groups: hard keratins, which are embedded in cystine-rich proteins, and soft keratins which form loose bundles and offer mechanical resilience to epithelial cells. Keratin monomers have the ability to self-assemble and polymerize into porous, fibrous scaffolds with cell binding motifs that can create a three-dimensional matrix that allows for cell attachment and proliferation.

Hair-derived keratin hydrogels loaded with either/or in combination of IGF-1 and fibroblast growth factor have had a positive impact on muscle regeneration following VML injury. Implantation of these fibers resulted in significantly greater functional recovery (in both histological and contractile force analyses) over untreated muscle 8 weeks post-injury (Passipieri et al. 2017; Baker et al. 2017).

Oxidatively extracted keratin and reductively extracted keratin, keratose, and kerateine, respectively, have also been tested as therapeutic candidates for VML injuries. Though these materials possess different structural, chemical, and mechanical profiles, both keratin derivates offered protection to deliverable skeletal muscle progenitor cells and growth factors. Additionally, keratin-based biomaterials elicit minimal inflammatory and immunological response while increasing expression of muscle-specific markers (Tomblyn et al. 2016).

Fibrin

Fibrin is a protein produced from the cleavage of fibrinogen (Brovold et al. 2018). It has strong adhesive properties and promotes cell attachment and angiogenesis. Cell-laden fibrin microthreads stimulated the formation of skeletal muscle fibers, connective tissue, and Pax7-positive cells. These constructs decreased pathologic deposition of collagen in the wound and promoted the growth of new tissue. Moreover, the implantation of these cell-carrying microthreads into the site of injury restored contractile force to the muscle (Page et al. 2011).

Other reports of cell-laden fibrin microfibers implanted into VML defect sites had increased expression of total regenerating muscle marker embryonic MyHC and adult MyHC isoforms. The combination of material and adipose-derived stromal cells increased muscle reconstruction and qualitative muscle parameters in vivo in the VML defects (Gilbert-Honick et al. 2018a), while cross-linked fibrin loaded with hepatocyte growth factor significantly enhanced the force production of muscle tissue 60 days after injury, as well as enhancing myoblast differentiation and angiogenic response (Grasman et al. 2015).

6.5.4.2.2 Polysaccharides

Chitin and Chitosan

Chitin is an abundant polysaccharide that is found on the exoskeleton of arthropods and the cell wall of fungi (Brovold et al. 2018). It is biocompatible and biodegradable, and has been shown to support cell attachment and proliferation due to its porous nature and abundance of physical cues. Chitosan is the fully or partially N-deacetylated derivative of chitin and shares many of its biocompatibility characteristics with its progenitor.

Previous studies have undertaken the creation of self-healing N-carboxyethyl chitosan injectable hydrogels able to encapsulate either C2C12 myoblasts or human umbilical vein endothelial cells (HUVEC) (Guo et al. 2019). These constructs not only maintained the viability and promoted the proliferation of encapsulated cells but increased the number of centrally localized myonuclei in TA muscle at 1 and 4 weeks following VML injury.

6.5.4.2.3 Glycosaminoglycans

Hyaluronic Acid

Hyaluronic acid (HyA) is a disaccharide found in most connective tissues, synovial fluid, and vitreous of the eye (Brovold et al. 2018). It is easily chemically modifiable, viscoelastic, hydrophilic, biocompatible, and biodegradable. Studies have evaluated the additive effect of a hyaluronic acid and laminin hydrogel as a myoconductive scaffold co-delivered with minced muscle grafts (Goldman et al. 2018). While HyA gels did not offer any improvements in peak tetanic force production over minced muscle grafts, the use of HyA hydrogels has been shown to improve strength over no treatment (Dienes et al. 2021). Additionally, HyA hydrogels functionalized with the laminin peptide IKVAV can potentially enhance migration, proliferation, and muscle-specific transcription factors in myoblasts (Silva Garcia et al. 2019).

6.5.4.2.4 Cellular Matrices

Decellularized Matrices (Biomimetic Scaffolds)

Decellularized and acellular matrices or scaffolds are some of the most commonly used regenerative therapies in VML injuries. Decellularized matrices are derived from tissue explants or ECM that has been trypsinized and washed in detergent to eliminate any cells. What is left behind is the structural proteins which can then become a platform for cell culture to deliver live, proliferating cells into the injury defect or for use on its own to stimulate the growth and differentiation of progenitor and satellite cells (Wolf et al. 2015; Goldman et al. 2020). These materials have the advantage of being biocompatible and easily integrated to the host injury system.

Previous research has reported mixed results on the success of these scaffolds as delivery systems or structural templates following VML injuries. Earlier reports saw a shift in muscle healing response from deposition of scar tissue in the VML defect to the remodeling of the muscle utilizing the ECM scaffold (Sicari et al. 2012). Further investigations have demonstrated the use of acellular scaffolds to create a pro-myogenic environment that supported the de novo formation of muscle fiber (Perniconi et al. 2011) and improvements in force production of the TA muscle after transplantation of muscle-derived ECM despite the exacerbated production of fibrotic tissue (Corona et al. 2014).

On a more physiologically relevant note, one clinical study used electrodiagnosis to evaluate the effectiveness of ECM scaffold implantation in VML patients. Baseline measurements were compared before and 6 months after implantation. In 5 out of 8 patients, the implanted scaffolds had electrical activity, suggesting enervation. This increase in electrical activity was seen in combination with an improvement of muscle strength (Han et al. 2016).

Minced Muscle Grafts

Based on the mixed success of the use of decellularized matrices as biological scaffolds, various researchers have suggested the use of homologous scaffolds for skeletal muscle regeneration. Skeletal muscle-derived ECM would provide an environment similar to that missing within the tissue defect of VML injuries, secreting all the necessary growth factors and signaling molecules required for myofiber growth. Studies from various labs report improvement in muscle generated contractile force with minced muscle graft treatment following VML injury (Chen and Walters 2013; Corona et al. 2013a; Corona et al. 2017a; Ward et al. 2016). VML injuries treated with minced muscle grafts also display improvements in histological muscle quality and weight and increased myogenic and regenerative markers (Corona et al. 2013a; Corona et al. 2017a; Corona et al. 2017b; Kasukonis et al. 2016).

6.5.4.3 Synthetic Biomaterials

The early twentieth century saw the invention of synthetic polymers. These composites gave way to a brand-new field of materials for use in medical applications. Compared to naturally derived materials, these polymers are relatively easy and inexpensive to produce while maintaining low batch-to-batch variability. These synthetic polymer materials have consistent mechanical, chemical, and structural properties and are readily tailored to vary stiffness and degradation kinetics (Sarkar et al. 2017). As the synthesis and creation of synthetic biomaterials has evolved, more alternatives of host-integrative, biocompatible, and biomimetic have arisen. Some of these have been used successfully as therapeutics in VML injuries.

6.5.4.3.1 Poly(Glycolic Acid) (PGA)

PGA is a biodegradable polyester typically synthesized by the polycondensation of glycolic acid (Sarkar et al. 2017). It is commonly used for its fast degradation kinetics, which yield acidic degradations products that are thought to induce inflammation. This material can induce localized immune response from effector cells due to its degradation products, though there is no consensus on whether this response hinders tissue regeneration. Saxena et al. reported on the use of PGA meshes seeded with myoblasts as a means to engineer three-dimensional skeletal muscle tissue (Saxena et al. 2001). They demonstrated for the first time the ability of myoblasts to survive in vivo in the absence of mature skeletal muscle tissue.

6.5.4.3.2 Poly(Lactic-Co-Glycolic Acid) (PLGA)

PLGA is an ester copolymer derived from PGA which incorporates lactic acid monomers with glycolic acid monomers by means of ring-opening

co-polymerization synthesis process. It is hydrolytically biodegradable and biocompatible, elicits low toxicity, and is one of the most used synthetic biomaterials for skeletal muscle applications (Wolf et al. 2015). The use of PLGA-myoblasts constructs induced the formation of multinucleated myotubes in a study by Kamelger et al. Implanted constructs showed light fibrovascular growth without inflammation (Kamelger et al. 2004). Another study by Narayanan et al. developed an aligned electrospun fiber scaffold using PLGA that enhanced alignment, growth, and differentiation of myoblasts, in vitro, and resulted in the formation of dystrophin-positive myofibers in a TA muscle model of Duchenne muscular dystrophy (Narayanan et al. 2020). Their research on how these fiber scaffolds enhance skeletal muscle regeneration in VML injuries is ongoing.

6.5.4.3.3 Polycaprolactone (PCL)

PCL is a biodegradable polyester approved by the FDA for use in drug delivery devices and sutures. In tissue engineering, PCL is often used due to its favorable mechanical properties and slow degradation kinetics (Wolf et al. 2015). Aligned PCL nanofibers have shown promise as therapeutics for VML injuries. Muscle defects treated with these fibers have shown to have an increase in regenerating myofibers, myogenic protein expression, and HSP-70 expression, as well as an overall increase in muscle mass over non-treated controls. Moreover, VML defects treated with PCL fibers exhibited decreased CLN expression at 28 days after injury, suggesting that the scaffold decreased the severity of injury and provided mechanical support to the defect site (Patel et al. 2019).

6.6 Regenerative Rehabilitation

The status quo as it pertains to treatment strategies for VML is (1) various regenerative medicine approaches that modestly improve function preclinically and require further maturation to achieve clinically meaningful functional improvement; (2) a dearth of VML studies that include reliable, clinically relevant outcome measurements of function; and (3) no standards of care for the patient. Regenerative rehabilitation offers a departure from the status quo by combining best practices of regenerative medicine and rehabilitation, especially if future studies incorporate clinically meaningful outcome measurements of muscle function. Table 6.1 provides examples of rehabilitation and regenerative medicine studies that included at least one Tier 1 (Fig. 6.3) functional outcome comparison between VML-injured with no intervention and VML-injured with intervention. This provides context to discuss next steps toward advancing regenerative rehabilitation for VML and pursuing standards of care for patients with VML injuries.

Table 6.1 (note references within) was adapted and expanded upon from a systematic review and meta-analysis published in 2019 (Greising et al. 2019). This

Table 6.1 Summary of select rehabilitation and regenerative medicine study outcomes

Approach	Tier 1 functional outcomes	Percent Δ to VML-injured, no treatment	Tier 2–Tier 4 supporting outcomes
Rehabilitation			
Electrical stimulation plus passive range of motion (2xWk, 1-4mo duration) (Greising et al. 2018)	Peak-isometric torque; passive range of motion; mitochondrial OCR	+30% −32% NC	Tier 2: None; Tier 3: Fibrosis, inflammation; Tier 4: None
Wheel running (Southern et al. 2019)	Peak-isometric torque, mitochondrial OCR	+42% NC	Tier 2: Mitochondrial complex content/enzyme activities; Tier 3: Vascularity; Tier 4: Voluntary wheel running
Voluntary wheel running (1 or 7 weeks) (Aurora et al. 2014)	Peak-isometric torque normalized to body weight and peak-isometric torque of tibialis anterior (with and without EDL)	With EDL +9% Without EDL +17%	Tier 2: Muscle fiber number/cross-sectional area, histological evidence of regeneration; Tier 3: Fibrosis; Tier 4: None
Regenerative medicine			
Gene overexpression (PGC1) (Southern et al. 2019)	Peak-isometric torque, mitochondrial OCR	+54% +41%	Tier 2: Mitochondrial complex content/enzyme activities; Tier 3: Vascularity; Tier 4: Voluntary wheel running
Autologous mince muscle graft (Ward et al. 2016)	Peak-isometric torque	+35%	Tier 2: None; Tier 3: Inflammation; Tier 4: None
Scaffoldless multiphasic muscle units (VanDusen et al. 2014)	Peak-isometric force	+36%	Tier 2: Muscle fiber number/cross-sectional area; Tier 3: None; Tier 4: None
Acellular matrices + MSCs (Qiu et al. 2018)	Peak-isometric torque	+67%	Tier 2: Muscle fiber formation; Tier 3: Fibrosis, inflammation; Tier 4: None
Bladder acellular matrices + MDCs (Corona et al. 2012)	Peak-isometric force	+61%	Tier 2: Satellite cell content/dynamics; Tier 3: None; Tier 4: None

(continued)

Table 6.1 (continued)

Approach	Tier 1 functional outcomes	Percent Δ to VML-injured, no treatment	Tier 2–Tier 4 supporting outcomes
Muscle-derived ECM + MSCs (Corona et al. 2013b)	Peak-isometric force Peak-isometric torque	NC NC	Tier 2: Muscle fiber number/cross-sectional area; Tier 3: None; Tier 4: None
Bladder acellular matrices + MDCs (Corona et al. 2014)	Peak-isometric torque	+26%	Tier 2: None; Tier 3: None; Tier 4: None
Porcine urinary bladder matrix (Aurora et al. 2015)	Peak-isometric torque	8 weeks +29% 16 weeks NC	Tier 2: Satellite cell content/dyanmics; Tier 3: Fibrosis, vascularity; Tier 4: None
MPCand growth factor-loaded keratin hydrogels (Baker et al. 2017)	Peak-isometric torque	Keratin alone = +31% Keratin+IGF-1 = +20% Keratin+bFGF = −6% Keratin+IGF-1 + bFGF = +61%, Kertatin+MPC = +3% Keratin +MPC + bFGF = +15% Keratin+MPC + IGF-1 = +28% Keratin+MPC + IGF-1 + bFGF = 2%	Tier 2: None; tier 3: Fibrosis, histological evidence of regeneration; Tier 4: None
Acellular scaffold (Greising et al. 2017)	Normalized-isometric torque	NC	Tier 2: Histological evidence of regeneration, satellite cell content/dynamics; Tier 3: Inflammation; Tier 4: None
Anti-fibrotic (losartan) (Garg et al. 2014a)	Peak-isometric force	−17%	Tier 2: Histological evidence of regeneration, satellite cell content/dynamics, Tier 3: Fibrosis, inflammation, lipid content; Tier 4: None
HGF loaded, cross-linked fibrin microthread scaffolds (Grasman et al. 2015)	Mean twitch force, force recovery: injured ratio	+25%	Tier 2: Satellite cell content/dynamics, histological evidence of regeneration; Tier 3: Vascularity, fibrosis, cell populations; Tier 4: None

(continued)

Table 6.1 (continued)

Approach	Tier 1 functional outcomes	Percent Δ to VML-injured, no treatment	Tier 2–Tier 4 supporting outcomes
Minced muscle graft (Aguilar et al. 2018)	Peak-isometric force	+64%	Tier 2: Histological evidence of regeneration; Tier 3: Inflammation; Tier 4: None
Muscle-derived ECM (Chen and Walters 2013)	Peak-isometric force	+14%	Tier 2: Muscle fiber formation, histological evidence of regeneration; Tier 3: None; Tier 4: None
Minced muscle graft (Corona et al. 2017a)	Peak-isometric force	NC	Tier 2: Muscle fiber number/cross-sectional area, histological evidence of regeneration; Tier 3: None; Tier 4: None
Muscle-derived ECM (w/ and w/o cells) (Garg et al. 2014b)	Peak-isometric force	w/o cells +16% w/ cells +35%	Tier 2: Satellite cell content/dyanmics, histological evidence of regeneration; Tier 3: Fibrosis, inflammation, cell populations; Tier 4: None
Laminin-111 supplemented hyaluronic acid-based hydrogel w/ minced muscle graft (Goldman et al. 2018)	Peak-isometric force	HA alone +17% HA + LMN +25% 50% MMG + HA +43% 50% MMG + HA + LMN +43%	Tier 2: Histological evidence of regeneration, satellite cell content/dynamics; Tier 3: Inflammation; Tier 4: None
Autograft (Li et al. 2014)	Peak-isometric force	4 weeks morselized = +5% whole = +1%	Tier 2: Muscle fiber number/cross-sectional area, histological evidence of regeneration; Tier 3: None; Tier 4: None
Prevascularized scaffolds with and without cells (Gilbert-Honick et al. 2018b)	Peak-isometric force	Acellular = +35% C2C12 = +32%	Tier 2: Histological evidence of regeneration; Tier 3: Fibrosis, cell populations; Tier 4: None
Tissue engineered muscle repair (Machingal et al. 2011)	Peak-isometric force	+64%	Tier 2: Muscle fiber number/cross-sectional area, muscle fiber formation; Tier 3: None; Tier 4: None

(continued)

Table 6.1 (continued)

Approach	Tier 1 functional outcomes	Percent Δ to VML-injured, no treatment	Tier 2–Tier 4 supporting outcomes
Progressive muscle cell delivery (Kim et al. 2016)	Peak-isometric force	+100%	Tier 2: Muscle fiber formation, histological evidence of regeneration, innervation; Tier 3: Vascularity; Tier 4: None
Decellularized + minced muscle autograft (Kasukonis et al. 2016)	Peak-isometric force	+38%	Tier 2: Histological evidence of regeneration; Tier 3: Fibrosis; Tier 4: None

is an important study to frame the current regenerative rehabilitation field because the authors identified 2312 studies (search ended January 2019); however, once those studies were screened for the inclusion of outcome measurements of muscle function, only 44 studies remained for the meta-analysis (2%). The lack of VML studies including a clinically meaningful outcome measurement is a current challenge area for the field if providing standards of care is a goal for the future. Skeletal muscle represents ~40% of a typical individual's body mass and represents an important metabolic engine for basal metabolism, and collectively the primary job of muscle is to contract, produce force, and help move the body. Ultimately, some measurement of metabolism, contractility, and/or movement should be involved in VML studies validating a new approach to improve the pathology.

Studies highlighted within the *Rehabilitation* and *Regenerative Medicine* sections of Table 6.1 reflect strategies designed to enhance muscle function by taking into consideration the pathology of the disease. For example, Aurora et al. (2014) and Southern et al. (2019) tested the effects of voluntary wheel running on outcome measurements of muscle strength, metabolic function, fibrosis, and vascularity. These are prominent areas of VML-related pathology described above. In the *Regenerative Medicine* section of Table 6.1 there are several approaches attempting to replace the lost extracellular matrix. For example, Qui et al. created a decellularized ECM scaffold and then seeded it with mesenchymal stem cells. Peak-isometric torque was reported ~67% greater in the intervention group compared to a VML group with no intervention. A similar ECM replacement approach was conducted by Garg et al.; only their research study design tested ECM with and without endogenous cells. Peak-isometric torque was ~16% greater without cells and ~ 35% greater with cells compared to a non-intervention group. These studies exemplify a balanced approach to improving muscle function after VML injury by addressing a pathology and relying on clinically relevant outcome measurements of muscle function.

Table 6.2 Summary of regenerative rehabilitation study outcomes

Regenerative rehabilitation			
Approach	Tier 1 functional outcomes	Percent Δ to VML-injured, no treatment	Tier 2–Tier 4 supporting outcomes
Acellular biomaterial + wheel running (Quarta et al. 2017)	Gait Peak-isometric force	+27% +67%	Tier 2: Innervation, satellite cell content/dynamics; Tier 3: Fibrosis, vascularity; Tier 4: None
Stem cell amniotic membrane scaffold + HIIT on treadmill (Izadi et al. 2021b)	Peak-isometric force	+180%	Tier 2: Innervation; Tier 3: Fibrosis, vascularity; Tier 4: None
Autologous mince muscle graft + wheel running (Corona et al. 2013a)	Peak-isometric force	+30%	Tier 2: Satellite cell content/dynamics, muscle fiber formation, innervation, muscle fiber number/cross-sectional area; Tier 3: Fibrosis, inflammation; Tier 4: Voluntary wheel running
Porcine urinary bladder matrix + voluntary wheel running (Aurora et al. 2015)	Peak-isometric force	+17%	Tier 2: None; Tier 3: None; Tier 4: None
HIIT training and decellularized human amniotic membrane scaffold (Izadi et al. 2021a)	Peak-isometric torque	+103%	Tier 2: None; Tier 3: Vasculature; Tier 4: None
Scaffold and mechanical loading (Dziki et al. 2018)	Peak-isometric force	+137%	Tier 2: None; Tier 3: Inflammation; Tier 4: None

Despite the gains in muscle function with rehabilitation or regenerative medicine noted in Table 6.1, further development of these approaches is required for full recovery. Statistically significant improvements in muscle strength, for example, range from 3 to 100% with intervention compared to a VML injury with no intervention (Table 6.1). However, a deeper interrogation of the data demonstrates these advances are still modest in terms of full recovery. For example, Kim et al. provided multiple injections of C2C12 myoblasts to VML-injured muscle and reported that muscle strength was 100% greater in treated vs. VML untreated rats (1.8 N kg^{-1} vs. 0.9 N kg^{-1}). But this significant improvement in strength pales in comparison to the strength of injury-naive controls (3.7 N kg^{-1}) highlighting the need for continued optimization of interventions for VML injury.

Regenerative rehabilitation seeks to take the best practices from rehabilitation and regenerative medicine alone and combine them to optimize restoration of muscle function after VML. To date, there have only been a few regenerative rehabilitation studies that included a clinically relevant outcome measurement of muscle function and a comparison to a non-intervention VML group (Table 6.2, note references within). The range of improvement in muscle strength is noteworthy in comparison

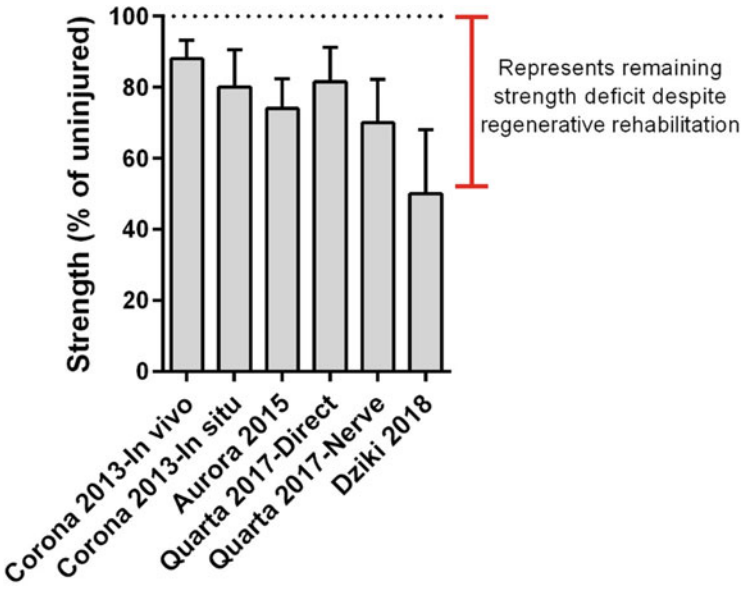

Fig. 6.5 Remaining muscle strength deficits from regenerative rehabilitation cohort of selected studies. Data are shown as a percentage of completely uninjured controls

to those in Table 6.1, 17–180%, and half of the studies report a gain above 100% compared to a non-intervention group. Though the sample size is small, these results support the notion that combined strategies can be more effective than rehabilitation or regenerative medicine strategies alone. That said, a comparison of the regenerative rehabilitation groups to uninjured cohorts, as opposed to VML untreated (Fig. 6.5), shows there is still significant room for improvement.

Beyond augmenting muscle function, these regenerative rehabilitation studies can also provide insights into the timing and/or absence of rehabilitation and the positive effects of combined approaches on other physiologically important outcomes. All of these studies, with the exception of Dziki et al., used a form of running as a rehabilitative approach (treadmill and voluntary wheel) (Dziki et al. 2018). Running stimulates adaptative responses through both mechanical and molecular cues (e.g., AMPK activation). Three studies (Aurora et al., Corona et al., Quarta et al.) initiated rehabilitation one week post-VML (Aurora et al. 2014; Corona et al. 2013a; Quarta et al. 2017), while Izadi et al. (Izadi et al. 2021b) started at two weeks post-VML. Noteworthy, Quarta et al. tested the amount of time required for VML-injured mice to return to their pre-injured daily running distances (~7 days) and noted that VML-injured mice that were forced to run daily on a treadmill immediately after injury had greater fibrosis than VML-injured mice that started forced treadmill running at 7 days post-VML (Quarta et al. 2017). They used this information to rationalize waiting at least one week to begin rehabilitation post-VML, although it should be noted that other studies have started rehab earlier with no reports of contraindicative effects (Greising et al. 2018; Southern et al. 2019). In

contrast to rehabilitation, Dziki et al. combined a bioscaffold regenerative medicine approach with hindlimb unloading that removes physical stimuli on the injured muscle and can be used to determine the effects of bedrest, inactivity, or sedentarism on muscle function post-VML (Dziki et al. 2018). Where an ECM bioscaffold improved muscle strength by 50% in VML-injured groups, hindlimb unloading completely reversed this effect. One conclusion that can be made from these studies is that there may be uncertainty on when to initiation rehabilitation, but the absence of physical stimuli, even that provided by basic ambulation, is detrimental. VML injuries in humans may indeed involve a period of recovery in which the patient is confined to a hospital bed or physically unable to voluntarily move. More clinical research is necessary to identify the extent to which assisted mobilization, rehabilitation, and other physical therapies can influence muscle function after VML.

Several of the studies noted in Table 6.2 included analysis of Tier 2–Tier 4 outcome measurements that are worth highlighting. Quarta et al. reported that rehabilitation resulted in a greater number of donor-derived myofibers being innervated following VML injury, and a greater frequency of mature NMJs in general (Quarta et al. 2017). Izadi et al. (Izadi et al. 2021b) noted that high-intensity interval rehabilitation combined with a bioscaffold and stem cell regenerative medicine approach produced greater vascular density compared to the regenerative medicine approach alone. And Corona et al. reported that voluntary wheel running in addition to an autologous minced muscle graft bolstered the immunoresponse and lessened markers of collagen deposition compared to the minced muscle graft alone. These studies exemplify how rehabilitation may improve the effectiveness of regenerative medicine approaches and be leverage to optimize functional recovery. Next, we highlight known areas for potential advancement of the regenerative rehabilitation field.

There are several known or straightforward ways to advance regenerative rehabilitation of VML. First, several regenerative medicine approaches (like those highlighted in Table 6.1) have not been combined with a rehabilitation strategy. Validating the extent to which wheel running or neuromuscular electrical stimulation influences vascularization of a biomaterial scaffold or augments a growth factor intervention is entirely feasible. Rehabilitation strategies are often considered pleiotropic, having greater than one effect on skeletal muscle, and therefore are suitable for many regenerative medicine approaches. Second, several innovative regenerative medicine approaches have not been validated to improve the functional capacity of the VML-injured muscle. These approaches can easily be advanced to the next stage of therapeutic relevancy by including any of the Tier 1 outcome measurement described above. Third, researchers should consult with physical therapists about what rehabilitation strategies are being used in a clinical setting that may require evidence-based data for further development. Approaches such as passive movement of the injured limb, vibration platform therapy, ultrasound, or weight-supported treadmill walking could be rigorously tested in animal models to expand feasible rehabilitation approaches. These approaches could then be combined with regenerative medicines to advance regenerative rehabilitation.

Finally, researchers must continue to pursue the "*unknowns*" of VML pathology. There are still knowledge gaps in the field related to the pathology of the VML injury, the plasticity of the remaining muscle, and whole-body implications that may negatively influence functional recovery. Continued holistic exploration of VML will undoubtedly produce more targets for regenerative rehabilitation. There is also a dearth of clinical data on traumatic muscle injury and the short- and long-term effects on patient health, mobility, comorbidities, and quality of life. For example, one study in animals hints that VML injury can negatively affect whole-body metabolism, yet it is unclear if VML patients have metabolic syndrome, insulin insensitivity, or changes in metabolic rate. Future researcher–clinician partnerships will be required to meet the challenges of answering these unknowns to fully understand the patient's perspective of this devastating injury.

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