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Old and new biomarkers for volumetric muscle loss

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Abstract

Volumetric muscle loss (VML) impacts skeletal muscles and causes damage to associated tissues such as blood vessels and other structural tissues. Despite progress in the VML field, current preclinical approaches are often ineffective at restoring muscle volume. Additional research is paramount to develop strategies that improve muscle mass and function, while restoring supporting tissues. We highlight mechanisms that govern normal muscle function that are also key players for VML, including intracellular calcium signaling/homeostasis, mitochondria signaling (calcium, reactive oxidative species (ROS)/oxidative stress), and angiogenesis. We propose an integration of these processes within the context of emerging biomaterials that provide structural support for muscle regeneration. We posit that new biomarkers (i.e. myokines and lipid signaling mediators) may serve as sentinels of early muscle injury and regeneration. We conclude that as new ideas, approaches, and models come together, new treatments will emerge to allow the full rebuilding of skeletal muscles and functional recovery of skeletal muscles after VML.

Introduction

Volumetric muscle loss (VML) is the extensive damage and a quick loss of 20% or more of the total amount of muscle mass that results in significant and persistent impairment beyond the normal capacity of regeneration of the tissues. It typically occurs suddenly due to a traumatic or surgical event such as those experienced by soldiers wounded in combat missions or civilians injured in automobile accidents [1]. After acute trauma (primary injury), adjacent tissues are further damaged (secondary injury) by the mechanisms including enzymatic cellular death, ischemia/reperfusion injury, mitochondria injury and calcium ion (Ca^{2+}) influx injury, and so on (Figure 1) [2]. In addition to alterations in muscle tissue function, the extensive muscle loss associated with this condition overwhelms the muscles' endogenous ability to self-regenerate [3].

Moreover, VML also involves damage to nerves, tendons, bone, and blood vessels, all injured, impaired, or destroyed during the primary and/or secondary injury (Figure 1). The cumulative result of this polytraumatic event is chronic inflammation, fibrosis, scar formation, and functional debilitation [3]. As such, the restoration of function requires the

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Conflict of interest statement

Kerrie Downing, Rhonda Prisby, Venu Varanasi, Jingsong Zhou, and Zui Pan have no conflicts to declare. Marco Brotto is the partner founding member of Bioform Sciences, LLC, the makers of MusQuLexx[®], a topical muscle cream for muscle pain and inflammation.

regeneration of both muscle and the supporting tissues (blood vessels, nerves, etc.). Tissue engineering using biomaterials is critical to provide scaffolds for regenerating muscle and other supporting tissues. Current treatment strategies largely depend on the type of injury (laceration with maintained tissue vs. a bomb blast with loss of tissues), and there is no effective treatment for extensive muscle loss [1].

Although we recognize VML as an acute catastrophic syndrome followed by long-term sequelae and the many advances in this field of research and practice, this review is not intended to summarize the recent advances of VML research. Rather, it takes a different angle to revisit VML by considering its links to other muscle diseases with the main objective of borrowing insights from these other pathological conditions that can be insightful for new therapeutics for VML. To this effect, we discuss key cellular processes that occur in these diseases that are most likely shared with VML, along with new biomaterial concepts and recently proposed biomarkers.

Significant muscle volume loss, sometimes extremely progressive, is also observed in many diseases, such as cachexia [4], sarcopenia (particularly during frailty syndrome) [5], and neuromuscular diseases [6,7]. Muscle loss, regardless due to VML or other muscle diseases, presents similar pathophysiological mechanisms and undergoes similar regeneration processes.

Although the current definition of VML does not comprise these other muscle losses, this review article revisits the pathophysiology and regeneration process of VML by embracing other muscle loss diseases as a new category of VML. Our second major goal is to go beyond the recognition of the more traditional VML biomarkers and propose new biomarkers in VML for damage and regeneration evaluation.

Pathophysiology in muscle loss and regeneration

After the initial trauma, the pathophysiologic response to traumatic injury can lead to secondary injury of otherwise uninjured cells by several causes (Figure 1, right panel) [2]. These factors resemble much of the underlying etiology for muscle loss revealed in human and animal studies, such that occurs with disuse [8], denervation [9,10], fasting [11], lack of gravity and unloading [12], aging (sarcopenia), cancer (cachexia), and neuromuscular diseases such as amyotrophic lateral sclerosis and spinal muscular atrophy [6,7].

Although the abovementioned five pathologic responses occur rather rapidly in traumatic muscle injury, these processes usually proceed in much slower pace in other disease conditions. However, there are cellular and signaling similarities, and in some cases, such as certain stages of cancer cachexia, unloading, denervation, and fasting, the maladaptation can proceed rapidly as well.

After primary injury, skeletal muscle immediately begins the regenerative process. Skeletal muscle regeneration occurs through a complex process loosely classified into four phases: the hemostasis phase, the destruction/inflammatory phase, the repair phase, and the remodeling phase. This process begins with the clotting of the wound, removal of damaged cells via several mechanisms, recruitment of muscle stem cells or progenitor/satellite cells,

and rebalance of cell proliferation and differentiation. Activation of muscle stem cells or satellite cells then follows, along with the cell cycle exit, fusion of mononucleated myoblasts to regenerate myotubes and myofibers. However, achieving muscle regeneration with perfect functional restoration and minimal fibrosis or scarring is still a challenge for treating extensive VML. As such, examining general muscle loss and regeneration is important for mechanistic understanding of VML pathophysiology.

Ca²⁺ handling mechanism in muscle injury and regeneration

Most processes in both muscle injury and regeneration involve intracellular Ca²⁺ signaling [13–16]. As a ubiquitous second messenger, Ca²⁺ regulates many cellular processes, ranging from gene transcription to muscle contraction, from cell proliferation to apoptosis [15]. Ca²⁺ is especially important in maintaining the proper structure and function of skeletal muscle. Three major Ca²⁺ handling mechanisms control the homeostasis of Ca²⁺ in skeletal muscles; these include excitation–contraction coupling (EC-coupling) [17], excitation coupled Ca²⁺ entry [18], and store operated Ca²⁺ entry (SOCE) [19]. EC-coupling refers to coupling of external electrical excitation via activation of voltage-gated Ca²⁺ channels at the sarcolemma and transient explosive elevation of intracellular Ca²⁺ via opening of the ryanodine receptor/Ca²⁺ release channel on the sarcoplasmic reticulum (SR). When depolarization of skeletal muscle sustains, excitation coupled Ca²⁺ entry occurs and results in extracellular Ca²⁺ across the sarcolemma. Similarly, SOCE also mediates extracellular Ca²⁺ into muscle cells; however, its activation is triggered by reduction of the SR Ca²⁺ storage [15,17]. In this mechanism, the depletion of the SR stores due to activation of the ryanodine receptor or inositol trisphosphate receptor activates a Ca²⁺ sensor at the SR, leading to the opening of sarcolemma-located store-operated Ca²⁺ channels.

SOCE was originally identified in nonexcitable cells more than three decades ago, and its roles in skeletal muscle have been recognized much later [15,19]. It consists of two main components: stromal-interacting molecule 1 (STIM1) at the SR as a Ca²⁺ storage sensor and Orai or transient receptor potential cation channel subfamily C member 1 proteins at the sarcolemma as an SOCE Ca²⁺ channel. When there is a reduction in Ca²⁺ storage, STIM1 is activated and forms patches and induces the aggregation of Orai1, which further triggers the activation of SOCE, leading to the flow of extracellular Ca²⁺ into the cell. SOCE-mediated intracellular Ca²⁺ signaling is spatially temporally confined in the form of waves, spikes, or oscillations, and the latter often activates gene transcription. In particular, the frequency, amplitude, and duration of these intracellular Ca²⁺ oscillations compose the specific Ca²⁺ codes for cell proliferation, migration, and differentiation [20–25]. In skeletal muscles, Ca²⁺-dependent signaling cascades include the calcineurin–nuclear factor of activated T cells pathway, the Ca²⁺/calmodulin-dependent kinases–nuclear factor kappa B pathway, CaM-dependent kinases and phosphatases (calpain), and mitogen-activated protein kinases [26].

As mentioned earlier, an elevated intracellular Ca²⁺ level can cause muscle cell apoptosis in muscle diseases. The Ca²⁺ influx is usually mediated by SOCE [27]. Accumulating evidence shows that Ca²⁺ influx and its downstream signaling pathways are required during muscle regeneration, and the alterations in these pathways are associated with various muscle

diseases. Therefore, it is plausible that the alteration of SOCE machinery genes, such as Orai1, STIM1, and TRPC, at different stages of muscle regeneration after VML may serve as new biomarkers to evaluate tissue repair.

Mitochondria as a sensor of intracellular Ca²⁺ profiles in muscle damage and regeneration

Despite variations in the initial cause of damage, evidence highlights apoptosis as a major factor contributing to muscle loss [10,28–30]. Mitochondria, taking up 10–15% volume of mammalian skeletal myofibers [31], not only serve as the energy provider, but are also intimately involved in apoptosis [30,32–34]. Excessive mitochondrial reactive oxidative species (ROS) generation is revealed as an early event of the apoptotic process [35]. Although low level mitochondrial ROS serves as an essential signal regulating various physiological processes [36], excessive ROS production leads to oxidative stress, which is detrimental to cell function [37]. Indeed, prolonged muscle inactivity is implicated with excessive mitochondrial ROS production in myofibers [38–40], whereas mitochondria in contracting normal muscle do not show enhanced ROS production [41–43].

Although mitochondrial function in muscle is fine-tuned by intracellular Ca²⁺ signaling, mitochondria also have the ability to shape intracellular Ca²⁺ profiles in myofibers [44–47]. There is always a cross talk between mitochondrial Ca²⁺ and ROS signaling [48–50]. Cytosolic Ca²⁺ transients are spatiotemporally well-controlled Ca²⁺ release events from the SR in a myofiber responding to the motor nerve activation during EC-coupling. Skeletal muscle disuse induced by various pathological conditions including VML could partially or completely disrupt EC-coupling and eliminate cytosolic Ca²⁺ transients. What is the immediate response of mitochondria to the cessation of cytosolic Ca²⁺ transients in denervated myofibers? A study from Karam et al. [51] found that denervated myofibers lacking cytosolic Ca²⁺ transients exhibited frequent mitochondrial permeability transition (mPTP) opening and enhanced mitochondrial ROS production. Remarkably, restoration of cytosolic and subsequent mitochondrial Ca²⁺ transients by brief electric field stimulation immediately stopped mPTP opening and restored mitochondrial ROS production to the normal level within a minute. This suggests that dynamic mitochondrial Ca²⁺ transients induced during EC-coupling are vital to keep mPTP in a closed status in myofibers, which could be essential for optimal muscle repair and regeneration.

Another pathological hallmark of long-term muscle inactivity is a steady-state elevation of cytosolic Ca²⁺ levels [52–55], leading to overload of mitochondrial matrix Ca²⁺ ([Ca²⁺]_{mito}), inducing cell apoptosis through enhancing ROS production and increasing the release of proapoptotic proteins through mPTP opening [38]. Until recently, it remained elusive why an elevated cytosolic Ca²⁺ level is revealed in inactivated myofibers. A study from the Saez's Research Laboratory discovered that de novo expression of connexin hemichannels in the sarcolemma of myofibers is the upstream cause of the enhanced membrane permeability that subsequently leads to elevated cytosolic Ca²⁺ levels [52,56,57]. Therefore, mitochondria act as sensors of spatiotemporal profiles of intracellular Ca²⁺ in myofibers. Loss of physiological mitochondrial Ca²⁺ influx, in combination with steady-

state elevation of $[Ca^{2+}]_{mito}$, forms an important factor leading to further muscle loss in inactivated skeletal muscle in VML. It is possible that serum detection of ROS/reduction-oxidation (REDOX) along with mitochondrial DNA could lead to new directions to diagnose or evaluation of the efficacy of various treatments for VML.

Angiogenesis in muscle regeneration

After VML, the repair of thick, complex tissues, such as skeletal muscle, is challenged by the ability to create new blood vessels and restore the vascular supply [58], a prerequisite for tissue healing. Therapeutic approaches used to restore the loss of muscle mass and function include vascular grafts [59], synthetic and natural polymers [1], decellularized extracellular matrix [60], and bioactive glasses [61]. These therapeutic remedies often also attempt to regenerate the supporting tissues. For example, bioactive glass was effective at stimulating angiogenesis and the expression of genes related to muscle regeneration with *in vitro* experimentation, whereas the healing of VML corresponded to vascularization and the activation of satellite cells in *in vivo* studies [61]. Noteworthy with these findings is the fact that the bioactive glass was able to stimulate angiogenesis and muscle satellite cells in the absence of added stem cells or growth factors [61]. Our group found that this effect was also found with silicon oxynitride semiconductor biomaterials that release ionic Si. The release of ionic Si enhances the expression of antioxidants NRF2 and SOD1, myokines γ -aminobutyric acid (GABA) and NRTN, and genes MyoG and MyoD that stimulate cell viability under the aforementioned oxidative stress conditions [62].

Vascular constructs created with microvascular fragments from adipose tissue demonstrated angiogenic sprouting [63] and the capacity for perfusion in the newly created blood vessels [64]. Furthermore, the use of hydrogels containing microvascular fragments to treat VML augmented vascular density within the tibialis anterior muscle of rats versus the use of collagen gels devoid of cells or single-cell scaffolds [65]. *In vitro* analysis of constructs containing microvascular fragments and myoblasts elicited microvascular networks that were longer and more highly branched versus constructs containing only microvascular fragments [66], indicating the muscle itself participates in angiogenesis. Furthermore, two weeks after implantation into a large volumetric muscle defect created in the rat biceps femoris, a dense microvascular network capable of perfusion was observed in the vascularized collagen hydrogel seeded with myoblasts versus an empty construct [66]. However, total vascular volume within the muscle defect was similar between the vascularized hydrogels with and without myoblasts [66]. Although development of blood vessels capable of perfusion occurred under both circumstances, these constructs did not result in muscle regeneration 8 weeks after injury [66]. These preclinical experimental strategies aimed at restoring vascular development and function in concert with muscular regeneration and function are in their infancy but are promising. Given the heterogeneity of blood vessels, the various muscle fiber types, and issues associated with advancing age and/or disease, much work is needed in the development of these strategies to optimize recovery from VML in a wide range of patient populations. Strategies aimed at stimulating early angiogenesis should provide fertile ground for VML repair. In addition, advanced *in vivo* imaging techniques may assist, in the near future, in determining where tissues lack a sufficient vascular supply and are in need of angiogenic stimulation.

Biomaterials and tissue engineering approaches to enhance neuromuscular tissue regeneration

Because of the large volume depletion of muscle concomitant with neuromuscular and vasculature components, it is necessary to use tissue engineering approaches that provide structural support during VML regeneration. Tissue engineering using biomaterials (including natural approaches like decellularized extracellular matrix and synthetic polymers such as polycaprolactone, poly-L-lactic acid, and poly-L-GLYCOLIC ACID) is needed to stabilize the resulting VML defects and offer a conductive path for revascularization coupled with neuromuscular regeneration. Biomaterials form the basis of tissue-engineered constructs and play critical roles during the processes of tissue regeneration. Each can be synthesized/fabricated in scaffolds, nanoparticles, or thin film structures.

Provided the mechanical properties of the biomaterials can stabilize existing defects, stimulation of tissue regeneration then falls to the scaffold's chemistry, ideally enhancing neurogenic, myogenic, and angiogenic biomarkers. Such enhancement must endure as the tissue recovers from initial increased concentration of ROS (e.g. H_2O_2 and O_2^-) and oxidative stress. Biopolymers usually have surface functional groups that offer cell guidance or stimulatory function to facilitate tissue growth. For example, three-dimensional (3D) printed poly-L-GLYCOLIC ACID exhibited enhanced myogenic differentiation markers such as myosin heavy chain and myogenin [67]. From a fabrication standpoint, use of microfluidic-enhanced 3D bioprinting was able to align myoblast-laden hydrogels for functionally organized myofibers [68]. In an analogous aligned fiber fabrication study, basal lamina mimetic nanofibrous peptide networks enhanced myogenesis [69]. For neurogenesis and myogenesis, bioinspired 3D human neuromuscular junction development was enhanced in suspended hydrogel array [70]. Biopolymers such as gelatin and chitosan have also been used to increase the density of vascular tubules in human endothelial cells needed to form the basis for vascular supply to musculoskeletal (MSK) tissues. Modification of these materials using nanoparticles and multidomain peptides has been shown to augment these 3D constructs for tissue regeneration. Nanoparticles also play a key role in stimulating cellular activity. For example, gold and gold-silver alloy nanoparticles enhance the myogenic differentiation of myoblasts through the p38 mitogen-activated protein kinase signaling pathway and promote *in vivo* skeletal muscle regeneration [71]. Nanosilicate biomaterials that release ionic silicon (Si) have also exhibited proangiogenic responses in human primary endothelial cells under normal and toxic oxidative stress conditions. Zinc oxide nanoparticles promote the formation of myogenic differentiation into myotubes in mouse myoblasts [72]. The mechanical stimulation of adhesion receptors using light-responsive nanoparticle actuators enhanced myogenesis [72]. Furthermore, surface modification of biopolymers with multidomain peptides offers added functionality to promote adhesion and growth of cells and offers self-assembly as nanofibers that deliver bioactive molecules and support tissue regeneration [73]. For example, targeted axonal import peptides delivered functional proteins for motor neurons after peripheral administration [74]. These biomarkers represent the cascade of events that result in vascular and neuromuscular tissue regeneration. Reflecting on the abovementioned rationale, several key choices can be made to arrive at a series of biomaterials that can be used to regenerate

lost neuromuscular tissue. Furthermore, additional modifications could include the addition of specific growth factors with time-released functions or mRNA encapsulated nanoparticles that would target a very specific protein or pathway.

New biomarkers in VML regeneration

In the previous sections of this article, we brought together the fundamental and new potential roles in VML of intracellular Ca^{2+} , mitochondria, angiogenesis, and targeted biomaterials by attempting to draw cellular and signaling similarities from other pathologies and the fundamental process of muscle and adjacent tissues that are damaged in VML.

As emphasized in our introduction, our goal is not minimizing as an entity by itself but to view in a broader context, to offer perhaps new insights from a different vantage view.

In the last decade, our understanding of endocrinology and hormonal regulation and tissue-to-tissue communication has completely changed [75–77]. Adipose tissue, bones, skeletal muscles, and even tendons are all now considered secretory tissues. New animal models have been created to study these biochemical bone-muscle interactions.

Much progress also occurred in the area of analytical chemistry with the identification of several molecules broadly involved in tissue repair and regeneration, some specifically involved in muscle repair and regeneration via bone–muscle cross talk. Our group, studying bone-muscle biochemical cross talk, coined the term osteokine [78] to indicate specific hormone-like factors secreted by bone cells acting in muscle cells to modulate their function. Among some of the osteokines we are studying, PGE_2 is a lipid-signaling mediator that enhances myogenic differentiation [79] and proliferation [80] of muscle cells, whereas fibroblast growth factor 9 reduces myogenic differentiation, pointing perhaps to the yin-yang of this process; in that, some factors might counteract or balance the action of other factors leading to optimal musculoskeletal health. We also developed a quick, accurate, and sensitive screening method for the precise isolation and quantification of aminobutyric acid isomers and enantiomers [62]. This test allowed us to quantify for the first time the levels in different media and substrates of the isomers of $[\beta\text{-aminoisobutyric acid (D- and L-BAIBA)]$ as well as GABA, and so on [62]. BAIBA is secreted from muscle and converts white fat into brown fat and seems very important to protect muscles and individuals against insulin resistance [81]. We showed in mice that BAIBA acted as a boneprotective factor against the loss of bone induced by unloading. This appears to be mediated by the ability of BAIBA to protect osteocytes against cell death induced by ROS [82]. Using our recently developed method, we studied the serum of ~200 women with low and high bone mineral density, yielding correlations with bone mineral density and osteoporotic fracture. In serum, GABA and (R)-3-aminoisobutyric acid (D-BAIBA) have positive associations with physical activity in young lean women, and they might serve as early biomarkers for diagnosis and treatment of osteoporosis [62].

We are not suggesting that these are the only secreted factors with therapeutic potential for VML. In fact, there are many of other candidates, certainly other growth factors, and

regulators of muscle stem cell proliferation and myoblast and myotube formation, along with specific genes and factors for the other tissues.

Notwithstanding, based on the new theory of bone-muscle cross talk, we propose that myokines and osteokines might be a set of convenient and attractive biomarkers for VML muscle regeneration. These new set of biomarkers along with the aforementioned processes/ biomarkers (angiogenesis, calcium, and mitochondria) and the refinement of smart design of biomaterials could very well lead to a new way of treating VML in the near future.

Concluding remarks and future perspectives

This is an exciting time for VML research as recent advances in research methodologies, technologies, and complementary markers of muscle regeneration provide an unprecedented opportunity to develop more robust VML regeneration tracking and tissue engineering strategies to repair multiple tissues (i.e. muscle, neuron, and blood vessel) for all patients with VML. The loss of associated tissues (e.g. blood vessels) with VML needs also to be considered when developing strategies for muscle regeneration. In addition, a holistic approach where the process of muscle regeneration is globally viewed, and its key check points used as biomarkers of the regeneration itself, could bring new hope to those affected with VML (Figure 2). The addition of other specific biomarkers such as myokines and lipid signaling mediators as early and long-term biomarkers of muscle injury, muscle remodeling, muscle repair, and regeneration will lead to new therapies.

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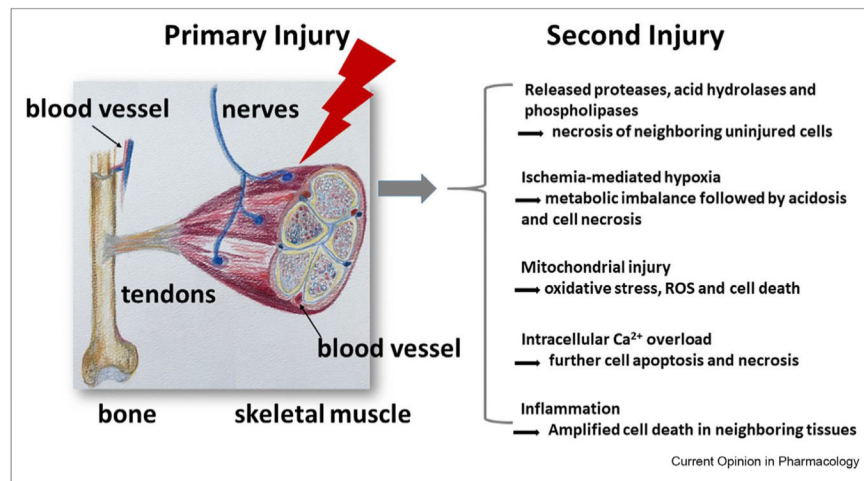


Figure 1. Primary and secondary injuries during VML. In addition to skeletal muscle, nerves, tendons, bone, and blood vessels are all injured, impaired, or destroyed during initial injury, which further cause second injury in several ways. These pathological conditions in second injury have been observed in other muscle diseases.

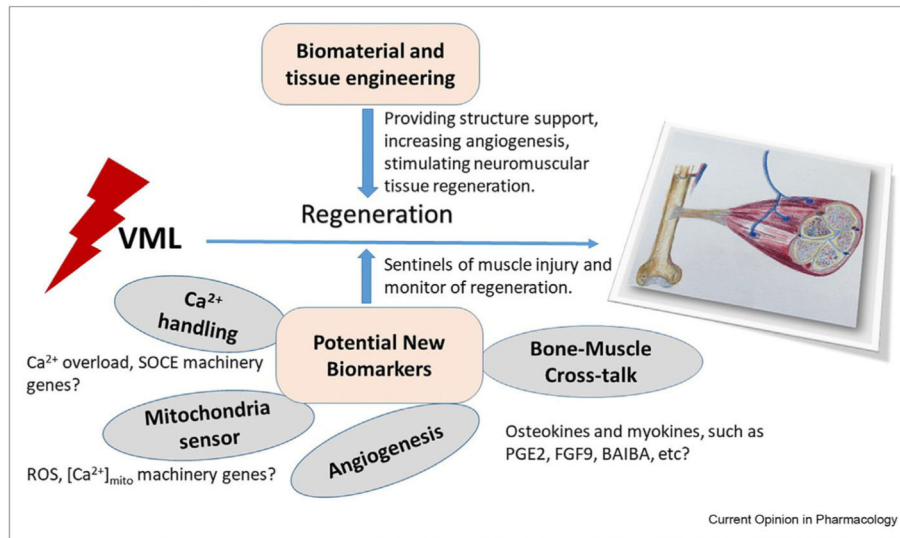


Figure 2. Potential new biomarkers in VML tissue regeneration. During VML tissue regeneration, effective biomaterial and tissue engineering is essential for reconstruction of the complex functioning system, including skeletal muscles, bone, blood vessels, and nerves. New biomarkers can serve as sentinels of muscle injury and monitor the regeneration. Our understanding of cross talk between bone and muscle and the pathophysiological conditions in other muscle diseases will shed light to identify new biomarkers in VML.