Mechanical signatures and models of the bone marrow niche

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[H1]Abstract

The bone marrow is a complex tissue with distinct cellular and mechanical heterogeneity, serving as the primary site for haematopoiesis. Under certain conditions, such as on the onset of mutations to healthy cells or alterations to the environment, the bone marrow can also become the origin of haematological malignancies, often characterised by uncontrolled self-renewal of hematopoietic stem cells overproduction of immature progeny and remodelling of the tissue microenvironment. This remodelling alters the composition and mechanics of the extracellular matrix (ECM), facilitating the proliferation and metastasis of leukemic cells. The ECM's elastic and dissipative properties are hallmarks of both health and disease progression in different tissues. However, studying the bone marrow's mechanical properties is difficult owing to inaccessibility in situ. Advanced 3D bioengineered models offer an approach to recapitulate the bone marrow's intricate mechanical properties, though incorporate the bone marrow's elastic and viscous component remain a challenge. Understanding the physiological and disease-specific mechanical ECM signatures is crucial for advancing bone marrow research and developing new therapeutics. In this Review we explore the structure-function relationship of the bone marrow, emphasizing its complex mechanical behaviour and discuss bioengineered models that recapitulate the mechanical properties in the healthy and diseased bone marrow niche, stressing the importance of replicating ECM physiological and pathological mechanical signatures in the future.

[H1]Introduction

The bone marrow niche is a specialised microenvironment that houses rare hematopoietic stem cell (HSCs). HSCs are just 0.01 – 0.04% of the total bone marrow cellular population[1], but are responsible for haematopoiesis, the process by which the cellular constituents of blood are continually replenished throughout an organism's lifetime[2]. This process is regulated through the dynamic interplay of cell-intrinsic factors, such as transcriptional and metabolic pathways, as well as cell extrinsic cues, such as tissue mechanics, provided by the bone marrow niche microenvironment. For example, if HSCs are removed from the bone marrow niche they lose stem cell activity in a matter of days[3]. Additionally, the niche plays a role in the pathogenesis and chemoresistance of haematological malignancies[4].

The bone marrow microenvironment displays vast cellular[5] and physical[6] heterogeneity. HSCs reside in the bone marrow at a frequency of ~1:30,000[3], the remaining cellular component are comprised of HSC progeny (e.g. blood cells and immune cells), and non-hematopoietic cell types (e.g. stromal cells and vascular endothelial cells), all of which contribute to the balance of self-renewal and differentiation of HSCs in the niche. Variations in ECM composition within specific microanatomical locations within the bone marrow, such as close to the bone surface or surrounding the vasculature, contribute to distinct mechanical properties, including changes in stiffness and viscoelasticity[7–9]. Although the cellular and molecular changes in the bone marrow associated with health and malignancy have been extensively studied, an inability to study bone marrow in situ has limited our understanding of the accompanying physical and mechanical changes that occur during remodelling of the malignant niche[4,5].

Although animal models have driven our understanding of the bone marrow microenvironment, they do not faithfully recapitulate human biology, disease mechanisms and responses to treatments[10,11]. For example, differences between mouse and human haematological systems include the proportions of cell populations and surface receptors used to identify cells in the hematopoietic hierarchy [12]. These species-specific differences in fundamental biology have led to gaps in mechanistic knowledge, failure to convert preclinical findings into meaningful clinical outcomes and in some cases, severe clinical failures due to adverse effects of therapeutics not predicted by animals, including fatal or multi-organ failures in humans[13–15]. Additionally, the translational gap from animals to humans has far reaching implications, from wasted resources to large financial losses owing to late-stage attrition of drug candidates and ultimately fewer treatments reaching patients. Human cells and tissues from primary sources can be cultured in vitro, but traditional, often 2D, approaches are often too simplistic and insufficient in replicating the complexity of the bone marrow as they often lack incorporation of any

of the physical milieu critical for cell regulation in the microenvironment[16]. Advances in bioengineering enable the development of systems such as three-dimensional models, organoids and organs-on-chip, that support the complexity and heterogeneity required to recapitulate the bone marrow microenvironment with a high degree of biological fidelity[17]. These models have the potential to provide translational platforms to recreate and decipher human disease mechanisms and develop novel therapies that will be effective in humans.

In this Review we provide a fundamental overview of the bone marrow niche, including its cellular components, their functions, and the composition of the ECM. We then introduce the mechanical properties of this microenvironment, and the role of tissue mechanics in health and disease. We describe advances in bioengineering human models of the bone marrow microenvironment *in vitro*, that are unravelling HSC regulatory and disease-relevant mechanisms associated to tissue mechanics. Finally, we discuss bioengineered models and their progress and limitations toward translation.

[H1]The bone marrow

Haematopoiesis is the process by which the cellular constituents of blood are continually replenished throughout an organism's lifetime, including red blood cell and immune cell production. The hematopoietic system within the bone marrow comprises various blood and immune cell populations with highly specialised functions, such as oxygen transport and immunity[2] . These cells originate through well-defined differentiation from HSCs, which are are characterized by their ability to self-renew and proliferate and ultimately give rise to mature blood cell types[18,19]. In humans, HSCs reside primarily in the bone marrow and in newborn infants, bone marrow is present and active in all bones. However, with age there is progressive conversion to inactive 'yellow' marrow and thus in adults, active bone marrow that continuously replenishes the blood system is primarily located in axial bones such as the craniofacial bones, sternum, ribs, pelvis and the proximal portion of the femur (Figure 1). It is estimated that an adult human generates ~4-5 $x10^{11}$ hematopoietic cells per day (0.5-1.5 x 10^9 in mice)[18]. To achieve this, the bone marrow must deliver the equivalent of half its total cellularity into circulation daily while maintaining a balance between self-renewal and differentiation. This process requires precise regulation from both cell-intrinsic and extrinsic regulation, facilitated by the complex bone marrow niche microenvironment. The concept of the niche refers to the network of cell-cell interactions necessary for maintaining stem cell self-renewal[19] and the bone marrow niche is a major complex multicellular organ that provides the molecular and physical cues essential for haematopoiesis[20]. A range of non-hematopoietic cell types, long-range humoral and neural signals, as well as the heterogenous ECM have all been identified as critical regulators of the bone marrow microenvironment for both normal and malignant HSC regulation[4,5].

Haematological malignancies are a collective term of neoplastic diseases, characterised by the overproduction of immature progenitor cells specific to the subtype of disease. Aberrant haematopoiesis and haematopoietic malignancies, such as myelodysplastic syndromes, myeloproliferative neoplasms and leukaemia's and lymphomas, are a major healthcare burden world-wide with average treatment costs for haematological malignancies are approximately two times higher per patient compared to other cancers[21]. Chemotherapy followed by bone marrow transplantation is a curative therapy for these diseases and involves the replacement of the hematopoietic and immune system with a donor bone marrow[22]. However, HSCs are often lost during chemotherapy due to off-target effects and the population of long term-HSCs required to repopulate bone marrow for the lifetime of an organism represent only a small fraction (~0.01-0.04%) of the total mononuclear cells in the bone marrow[23]. As a result, the number of available donor HSCs is often insufficient for successful transplantation, and ex vivo expansion is needed. HSC expansion remains a major clinical challenge[3]; several culture systems that demonstrated successful HSC expansion in vitro or in animal models used cytokines[24] and small molecules[25–29], but they have since failed in clinical trials owing to a loss of HSCs able to engraft long-term in humans[30]. These systems lack the physical and mechanical components of the bone marrow microenvironment, demonstrating the importance of these cues inaintaining naïve HSCs. The native niche provides a protective environment for HSCs, allowing for substantial expansion, adaption and response to injury[5]. Therefore, understanding the biomechanical mechanisms that govern HSCs in the bone marrow microenvironment is critical for the development of effective expansion protocols and identification of therapeutic targets.

[H2]Distinct bone marrow niches

The bone marrow is comprised of several hematopoietic and non-hematopoietic cell types that are interconnected by a vascular and innervated network[31]. The hematopoietic cellular constituents of the bone marrow in both humans and animal models have been identified primarily using defined combinations of cell surface markers, that identify blood and immune cell subsets using flow cytometry,[32] which indicates that most cells in the bone marrow space are differentiating or fully mature hematopoietic cell types, predominantly of the myeloid lineage. This myeloid prevalence is up to two times higher in human bone marrow compared to murine, which is enriched with lymphocytes [33,34]. The most primitive HSCs and multipotent progenitors that sit at the apex of this lineage hierarchy comprise only a minor fraction of the total bone marrow cellular content [4,35]. Several types of HSCs with progenitor-biases (lymphoid versus myeloid) or cell cycle states (quiescent versus activated) have been identified and are thought to preferentially localise to different regions of the bone marrow[36]. These regions are micro-anatomical niches, defined by their location or the type of blood vessels they

contain. For example, the central bone marrow cavity contains the central sinus, carries deoxygenated blood and gives rise to smaller vessels that form the sinusoidal niche. In contrast, in the arteriolar niche oxygenated blood is carried through small arterioles that sit near the bone surface of the bone marrow (Figure 2).

In addition to differences in the hematopoietic population, there is vast heterogeneity within the non-hematopoietic components comprising each micro-anatomical niche. Endothelial cells lining vasculature[37], osteoblasts[38,39], adipocytes[40], and subpopulations of mesenchymal stromal cells [41–43], all differ in localisation and role within the distinct niche compartments[5]. However, the specific functions and locations of arteriolar and sinusoidal niches remains contentious. Different studies over the last several decades have yielded conflicting findings, primarily due to the different transgenic mouse models used [4,5]. Additionally, differences in human and mouse bone marrow cellularity[34] and ECM[44] alongside the focus on flow cytometry-based identification of the hematopoietic composition of the bone marrow, often neglects the contribution of the dense, difficult to isolate, non-hematopoietic stromal networks meaning our mechanistic understanding of niche function is lacking -----[18].

Despite these challenges, transgenic mouse models have played an invaluable role in advancing our understanding of the bone marrow microenvironment due to its in situ inaccessibility in humans. With the continuous development of novel imaging techniques[31,45–47] and advances in spatial omics approaches[48,49], our ability to obtain direct information on human bone marrow is reducing the need to extrapolate murine biology to the human setting. Consequently, our understanding of the contextual bone marrow niche is continually evolving.

[H3]Arteriolar niche: The arteriolar niche is found at the interface of the bone and bone marrow, called the endosteum. It is covered by layers of bone-forming osteoblasts, bone-resorbing osteoclasts and mesenchymal stromal cells -derived osteoprogenitor cells[5]. The honeycomb structure of trabecular bone provides a large he endosteal surface area that is densely populated with small arterioles, sitting within <20 µm of the endosteum[37], resulting in higher oxygen pressure (pO_2: 2.9%) compared to the central bone marrow cavity (pO_2: 1.3%)[50]. The arteriolar niche comprises <10% of total bone marrow volume and approximately 15% of all HSCs[4]. Additionally, the arteriolar niche is thought to house long-term HSCs and transplanted HSCs isolated from this region show greater ability to home, or migrate, to the bone marrow, lodging in the arteriolar niches , and lead to increased long-term in vivo reconstitution compared to central marrow counterparts[51,52].

Quiescence, a reversible state in which a stem cell does not divide but retains the ability to re-enter proliferation, is essential to preserve HSC self-renewal capacity and protects HSCs from genotoxic stress. Early studies identified several different cell types or extracellular signals within the bone

marrow microenvironment thought to be the key factors regulating HSC quiescence. However, advances in new approaches, such as new genetic models and 3D imaging, have challenged these understandings revealing conflicting evidence regarding the roles of specific cell types and signals. This evolving perspective highlights the complexity of defining the bone marrow microenvironment, and understanding HSC regulation. Several For example, osteolineage cells were the first bone marrow cells identified as regulators of HSC dynamics as studies observed that transplanted HSCs were enriched at the bone surface post-transplantation.[53,54] Furthermore, human osteoblasts support HSC expansion in vitro[55] and in vivo murine studies have correlated the number of osteoblasts with the number[38,39] of HSCs, demonstrating that conditional ablation of osteoblasts leads to aberrant hematopoiesis suggesting a direct role for osteoblasts in regulating HSCs[56]. Extracellular signals including adhesion molecule N-cadherin, which mediates cell-cell interactions between osteoblasts and HSCs[38], and regulatory cytokines secreted by osteoprogenitors, such as CXC-chemokine ligand 12 (CXCL12)[57–60] and stem cell factor (SCF)[61], have all been implicated in HSC regulation, primarily through retaining the HSC pool within the niche. However, advances in 3D imaging, such as whole-mount confocal immunofluorescence imaging, have revealed that endogenous HSCs are not significantly associated with osteoblasts[42,45] and instead suggest that osteolineage cells do not directly maintain HSCs using N-cadherin[62–64] and are not a main source of CXCL12 and SCF[43,64,65].

Due to these 3D imaging studies of whole intact bone marrow, it is now understood that HSCs preferentially localise to the small arteriolar vasculature perfusing the endosteum, rather than being confined to the bone surface[66]. This arteriolar niche contains a subpopulation of mesenchymal stromal cells enriched for niche factor expression. in vivo murine studies have demonstrated that Nestin-GFP+ stromal cells are enriched within the arteriolar niche, these nestin-GFP+ MSCs express high levels of HSC regulatory factors CXCL12 and SCF[60,67,] and associate tightly with HSCs in the bone marrow,[60,67,68]. They overlap with a rare population of NG2+ expressing perivascular stromal cells that are associated with arterioles and localise with a population of quiescent HSCs (Figure 2).

[H3]Sinusoidal niche. Sinusoidal vessels are located in the central marrow cavity and form the second vascular niche. The sinusoidal niche houses over >90% of total active bone marrow and is responsible for daily blood production. Approximately 85% of HSCs reside in the sinusoidal niche; these are short-term HSCs, meaning this is an active population with limited self-renewal capacity and give rise to progenitor cells upon differentiation [37,69]. Endothelial cells lining sinusoids are more permeable than those lining arterioles and expose HSCs to deoxygenated blood plasma to promote differentiation, migration and egression of HSCs and progeny out of the bone marrow[37].

A subset of SCF-expressing mesenchymal stromal cells wrap around the sinusoids and regulate HSC activity. These stromal cells are nestin^{low}, but express high level of the adipo-osteogenic regulator *Lepr* (Leptin receptor)[41,65]. LEPR^{+} stromal cells overlap with a subset of stromal cells that express high levels of CXCL12, named CXCL12 abundant reticular (CAR) cells, which spatial transcriptomics of bone marrow from mice has identified as an Adipo-CAR subpopulation as they express more adipocyte-lineage genes compared to CARs more closely associated with arterioles, highlighting the differences in cell contributions between the two microanatomical niches [70](Figure 2).

In addition to mesenchymal stromal cells, other non-hematopoietic cell types, such as osteoblast and endothelial cells, contribute to the complex array of growth factors, chemokines and cytokines comprising the soluble niche cues that regulates HSC behaviour[71]. For example, endothelial cell-derived SCF signalling through the HSC c-Kit receptor has been implicated in HSC maintenance[65,72,73], demonstrated through selective deletion of *Scf* from endothelial cells in leading to a reduction in functional HSCs in murine models[73]. Furthermore, CXCL12 secreted by endothelial cells, CAR cells, Nestin+ cells, Lepr+ cells and NG2+ cells, signals through the CXCR4 axis and is crucial for HSC retention, maintenance and homing in the bone marrow[37,43,64,67]. Additionally, differentiated HSC progeny, such as megakaryocytes and macrophages, contribute quiescence-promoting factors; for example, megakaryocyte secrete CXCL4 which, directly influences HSC quiescence through enhanced adhesion to nearby stromal cells[74]. Finally, HSCs and early progenitors actively participate in self-maintenance through autocrine signalling. For example, highly enriched human CD34+ HSCs isolated from the bone marrow have been shown to secrete self-regulating factors including SCF, TPO and ANGPT1, binding to their respective receptors on HSCs and early progenitor cells[75].

The identification of putative cell types contributing to the soluble bone marrow niche signals has primarily been driven by transgenic mouse models through targeted cell-ablation studies or by conditional deletion of niche factors, such as *Cxcl12* and *Scf*[43,64,67]. However, identifying the precise source of paracrine signals remains challenging owing to the complexity of the bone marrow microenvironment and compensation by other cell types.

[H2] ECM composition and mechanics

The ECM provides the structural and mechanical framework for the bone marrow and plays an integral role in regulating cell behaviour and tissue function. To actively regulate bone marrow cells, the ECM sequesters growth factors, chemokines and cytokines, thereby modulating their availability. This reservoir of soluble factors can then be rapidly mobilized in response to physiological needs including immune response, tissue repair or general homeostasis. The ECM of the bone marrow niche acts as a dynamic natural hydrogel composed of a complex network of extracellular molecules produced by

niche cells[7,9] which in turn modulates cellular activity. The most abundant proteins in the bone marrow ECM are fibronectin, collagens I-XI, laminin, tenascin, thrombospondin and elastin[76–79]. These proteins provide cell surface adhesion domains and contribute to the stability and tensile strength of the ECM through their cross-shaped or fibrillar network structuresFurthermore, proteoglycans and their large glycosaminoglycan side chains provide resistance and structure to the ECM as well as bind growth factors and other signalling molecules[8].

The diversity of cell types in each niche leads to a heterogenous distribution of ECM proteins across the bone marrow microenvironment. The distribution of niche ECM proteins across the bone marrow has been mapped and sorted into three main categories; those associated with the endosteal surface, vascular basement membrane proteins, and those comprising the BM parenchyma[31]. The ECM of bone is composed[80] primarily of hydroxyapatite and collagen (types I-IV and X)[81], and is fibronectin and vitronectin dense. In contrast, both arteriole and sinusoid basement membranes are enriched for laminin, whereas the bone marrow parenchyma is enriched for fibronectin (Figure 2)[31].

Differences in ECM protein distribution can affect HSC and stromal cell function. In vitro studies have shown that murine HSC lineage commitment can be influenced by the presence of different ECM proteins[82]. Similarly, ECM structural heterogeneity affects the matrix's mechanical properties, which in turn influence cell behaviour and phenotype within the niche[83].

Because accessing the bone marrow in situ for mechanical testing is difficult, [84] studies report inter- and intra-sample heterogeneity[85,86]. However, overall models of the bone marrow niche support a stiffness gradient created by heterogeneous distribution of ECM proteins and cell types within the niche, with a softer sinusoid and laminin enriched central BM (E' ~ 0.3-1kPa) that becomes increasingly stiffer toward the collagen and fibronectin dense arteriolar region (E' ~ 25kPa)[9]. Nonetheless, it is important to recognise the challenges of accurately measuring bone marrow mechanics and interpreting data from different studies. For example, porcine bone marrow samples measured with bulk rheology and nano-indentation showed an effective Young's modulus (E') ranging from 0.25 to 24.4 kPa at physiological BM temperature (35^{o}C)[86]. However, bone marrow from mice femurs was measured using Atomic Force Microscopy and data were fitted with two different models: the Hertz-Sneddon model which assumes purely elastic responses and gave a Young's modulus of 0.14kPa, in contrast to the two-element K-V model which assumes the environment is viscoelastic rather than purely elastic and gave a Young's modulus of 0.52kPa[85]. Intra-sample heterogeneity was specifically attributed to differences in structural components of the tissue, stemming from the variations in cell populations that deposit different amount and types of proteins at each 'sub-niche' along the stiffness gradient.. Variations in measurement methodologies and tissue sources impact our understanding of

bone marrow mechanics and highlighting the importance of new methods needed to measure the human bone marrow mechanics in situ (Box1).

The importance of stiffness-dependent variations on HSC and multipotent progenitors' function and phenotype has been demonstrated by various *in vitro* studies that have attempted to model this environment[84,87–89]. Gelatinbased scaffolds have flexible physical and or chemical crosslinking natures that enable investigation of different biomechanical patterns. For example, in murine HSCs cultured on gelatin 3D scaffolds with higher stiffness (70kPa) and larger pore size (80µm) reduced HSC differentiation and increased HSC hematopoietic function compared to scaffolds with lower stiffness (20kPa) and pore size (30µm)[87]. Additionally, matrix stiffness can influence multipotent progenitor phenotype as stiff matrices were shown to favour megakaryocyte spreading, intracellular contractility and fibronectin fibril assembly whereas soft substrates promoted proplatelet formation[84]. Furthermore, dimensionality and stiffness-dependent changes can also influence the expression of bone marrow mesenchymal stromal cell markers[88]. For example, SCF and CXCL12 expression of bone marrow murine stromal cells (BMSCs) was partially rescued when cells were encapsulated in 3D gelatin methacrylate hydrogels compared to when seeded in 2D hydrogels where the expression of both factors was significantly downregulated over time. However their expression decreased as 3D matrix stiffness increased, demonstrating that matrix stiffness also regulates stromal cell expression of HSC regulatory factors within the niche[88]. Although gelatin-based hydrogels lack specific biochemical cues and are limited by their inconsistent degradation and oxygen and nutrient diffusion rates, these studies indicate that mechanical cues from the matrix can regulate HSC phenotype and function directly through HSC-ECM interactions and demonstrate the role of mechanical homeostasis within the niche.

[H2]The niche's viscous behaviour

Most biological tissues, such as the brain, liver, kidney, spleen, pancreas, adipose and myocardial tissue, bone cartilage and tendon[90] exhibit elastic and viscous characteristics under deformation, a property known as viscoelasticity. Viscoelasticity allows tissues to transition from an initial elastic response akin to elastic solids, to a a time-dependent response when subjected to mechanical loading. ,[91–93] As such, viscoelastic solid materials, including tissues exhibit stress relaxation in response to a constant strain and exhibit creep in response to a constant stress. In fact, the viscoelastic nature of materials is quantified by measuring stress relaxation time (the time it takes for stress to relax to half its initial value ($\tau_{1/2}$)), creep deformation (the time it takes for strain to increase over time) and the loss tangent (tanδ; the ratio between loss modulus (G'') to storage modulus (G') which indicates the dissipative nature of a material. The latter is highly frequency-dependent, with the G' and G'' increasing with the frequency of the stress /strain.

Rheology is used to assed the bulk viscoelastic properties of a material as stresses and strain can be applied in shear or axial directions. Atomic force microscopy and particle-based microrheology can assess viscoelasticity at a microscale level[92,94]. A combination of these techniques have been used to measure the viscoelastic properties of tissues and reconstituted extracellular matrices (ECMs)[85,86,95].

Several studies have demonstrated the bone marrow's dissipative properties. For example, rheological measurements from intact yellow porcine bone marrow samples suggest a high degree of dissipation within the tissue, with the calculated loss tangent reaching 0.2-0.3 when measured at a frequency of 0.1 Hz at 37^{o}C[86]. Furthermore, when investigating femurs from a pool of young and skeletally matured mice, a mean bone marrow viscosity η of 0.3 kPa·s[85] was reported significantly higher than the viscosity η obtained by rheological measurements of other studies, (below 1 to approximately 100 Pa·s depending on the region tested)[96,97]. This difference was attributed to sample preparation, origin of sample extraction (outside the central bone marrow) and instrument limitations, further highlighting the difficulty in validating data across different techniques. In addition, creep times, τ, which describe the rate at which a material undergoes creep deformation revealed that the bone marrow exhibited a faster creep time τ (τ = 36s), and therefore more viscoelastic nature compared to that of the cortical bone (τ = 140s) surrounding the bone marrow. [85]Although, stress relaxation measurements of the healthy human bone marrow have not been reported (Box 2), stress relaxation measurements of human bone marrow obtained from fracture haematomas showed a half time ($\tau_{1/2}$) of 400 ± 60 s[98]. These relaxation times are comparable to other animal-derived soft tissues such as skin, muscle and liver[99,100].

Such studies indicate that the bone marrow microenvironment exhibits considerable time-dependent properties. Additionally, potential changes in the bone marrow's dissipative properties due to disease or ageing could affect the mechanical cues sensed by HSCs and stromal cells and alter their biological responses. As a result, understanding how the bone marrow's mechanical properties change, including its elastic and viscoelastic nature, in healthy and disease states is imperative to progress the basic understanding of the microenvironment and to point towards new therapeutic avenues that target the mechanics of the environment during disease with the aim of restoring them to the healthy tissue state.

[H1] Bone marrow microenvironment in disease

The remodelling of the bone marrow microenvironment is a key event during the development of blood malignancies, including leukemias, lymphomas, and myelomas[101,102]. However, in disease states it is unclear whether mutations in HSCs drive the remodelling of the bone marrow niche, thereby

supporting disease progression, or if transformations in stromal cells and the microenvironment, due to aging, lead to the onset of malignancies, making HSCs more susceptible to secondary mutations[103]. The two theories are not mutually exclusive and have been shown to share dysregulated mechanisms and signalling pathways, including changes in biochemical profiles of niche cells (e.g dysregulated cytokine and growth factor release) and ECM mechanical properties, such as stiffness. These alterations contribute to the transformation of the microenvironment into a leukemic-supportive niche that sustains the activity of leukemic stem cells (LSCs) at the expense of HSCs[102]. Although disease-induced mechanical changes and subsequent cell responses remain unclear, they could potentially reveal critical mechanisms and targets that can evolve our understanding of the healthy and pathological niche.

[H2]Changes in the biochemical profile

Both aging and disease states, induce changes in the secretome within the bone marrow that serve to remodel the microenvironment and can aid the initiation and progression of cancer. As myeloid malignancies, such as acute or chronic myeloid leukemia progress, the bone marrow niche shifts to a pro-inflammatory microenvironment[104]. For example, during the development and progression of disease there is a correlated increase in the secretion of multiple pro-inflammatory cytokines such as Tumour necrosis factor alpha (TNF-α), interleukins 1β, 6 and 8 (IL-β, IL-6 and IL-8) as well as lipocalin-2 from malignant and/or stromal cells of the niche[102]. More specifically, IL-1β and IL-6, produced by leukemic cells, create a paracrine feedback loop that leads to the increased expansion of myeloid cells and occasional depletion of Nestin+ stromal cells, the ones responsible for the maintenance of healthy HSCs [102,105].[106–109]Additionally, increased TNF-α secretion from patient-derived acute myeloid leukaemia (AML) cells inducesthe subsequent over-expression of E-selectin, a vascular adhesion protein that desensitises leukemic cells to chemotherapy, [110]. Furthermore, elevated TNF-α levels induce adhesion of AML cells to endothelial cells via CD44, a process important for malignant infiltration and potential metastasis. The interaction of hyaluronan with CD44 in the environment also contributes to LSC proliferation and homing [111].[112]. Furthermore, lasma samples from healthy individuals and patients with myeloproliferative neoplasms, such as polycythaemia vera, essential thrombocythemia and primary myelofibrosis have demonstrated that patients have comparatively higher levels of lipocalin-2 and TNF-α [106] . High levels of lipocalin-2 (eg. > 50ng/ml of lipocalin-2)contributes to a pro-inflammatory microenvironment because it promotes stromal cell proliferation and secretion of cytokines such as TGF-β1, vascular endothelial growth factor, bone morphogenetic protein-2 and osteoprotegerin, as well as secretion of collagen type I; all of which result in increased osteoblastic differentiation and fibrosis overtime[102,107]. Therefore, the pro-inflammatory microenvironment is a hallmark of bone marrow pathologies and is an important targetable pathway

for the development of therapeutic drugs for haematological malignancies. In fact, the clinical development of antibodies and receptor inhibitors targeting pro-inflammatory, pro-tumorigenic cytokines such as IL-6, TGF-β and VEGF has made significant progress, with various agents tested in clinical trials[113]. However, the application of cytokine-targeted therapies faces considerable challenges. Patient-dependent tumour heterogeneity complicates treatment efficacy, while adverse effects associated cytokine therapies, such as capillary leak syndrome associated with IL-2 treatment, pose additional hurdles that delay the use of these therapies in clinical practice.

In the diseased bone marrow the increased production of osteoblasts, further drives the development of a pro-inflammatory leukemic niche. For example, increased numbers of compromised osteoblasts that secrete high levels of IL-1β, TNF-α and TGF-β have been reported in myeloproliferative neoplasms[102]. Additionally, osteoblast expansion has been also linked to the presence of inflammatory myelofibrotic cells within the niche. These are cells that produce high levels of collagen and downregulate maintenance factors such as SCF, CXCL12, and angiopoietin 1 which are normally produced by healthy osteoblasts and contribute to the healthy HSC maintenance [102,114]. Furthermore, myeloid malignancies, such as primary myelofibrosis and acute myeloid leukemia lead to fibrotic processes including deposition of collagen fibres and deregulation of ECM components which alter the biomechanical nature of the tissue. Fibrosis within the bone marrow niche impairs the function of healthy HSCs and hematopoietic processes and concurrently enables LSC migratory ability resulting in LSCs metastasizing to different sites within the body with the highest incidence of metastasis found in the lymphatic system [115]..

AML has poor prognosis and high treatment failures owing in large part to the interaction of LSCs with the bone marrow microenvironment, largely mediated by the CXCR-4/CXCL12 signalling axis, that enables them to survive, proliferate and migrate outside the bone marrow niche to secondary tumour sites[102,116]. CXCL12 secreted by bone marrow stromal cells is sequestered in the bone marrow stroma and acts as a chemoattractant supporting HSC retention within the niche[64,117]. However, research using CRISPR-Cas9-based genetic mouse models and CXCR-4 antagonists, has shown that CXCL12 also promotes homing and survival of LSCs in the bone marrow[118–120]. CXCL12 binds to the CXCR-4 receptor which is highly expressed by most malignant cells in the bone marrow, keeping LSCs in close contact with both ECM and supportive stromal cells that then generate anti-apoptotic and growth-promoting signals[121]. In fact, high-throughput OMICs analysis validated that human LSCs present an increased production of proteins involved in their interaction with the ECM (e.g. fibronectin, osteonectin thrombospondin-1) as we as highly deregulated proliferation pathways including the PI3K/Akt and ERK1/2 cascades, that could enable LSCs to remain quiescent within the niche [122]. Other in vitro studies have reported that bone marrow-derived mesenchymal stromal cells from AML patients have

decreased production of the CXCL12 chemokine compared to those from healthy patients[123] which could contribute to the compromised maintenance of healthy HSCs in AML. Furthermore, a murine transgenic mouse model of chronic myelogenous leukemic (CML) has shown that CML mice demonstrate significantly decreased levels of CXCL12 compared to controls.[124]. This decrease was also correlated to reduced homing and retention of LSCs within the niche promoting the development of extramedullary disease. Taken together, these studies suggest that while CXCL12/CXCR4 interaction is vital for the homing of LSCs and remodelling of the niche, transient alterations in CXCL12 production during disease progression can also cause the migration of LSCs from the bone marrow and result in initiation of disease at secondary sites.

[H2]Changes in ECM composition

In haematological malignancies, alterations in the composition of the bone marrow stroma accommodate the needs and functions of LSCs[4]. For example, in myeloproliferative neoplasms mutations in HSCs can induce progressive fibrosis, characterised by the formation of scar tissue, and altered stroma fibres in the bone marrow[125]. Fibrotic remodelling results from the dysregulated secretion of ECM proteins by stromal cells and LSCs, leading to subsequent mechanical and architectural changes in the bone marrow ECM[101,126,127]. Specifically, primary myelofibrosis patient-derived biopsies have revealed an abnormally increased deposition of collagen I fibres and reticulin fibres as well as increased levels of lysyl oxidase (LOX; 44.6 ng/ml in PMF patient samples compared to 28.4 ng/ml in healthy ones) and LOX-mediated ECM crosslinking within the bone marrow compared to biopsies from normal bone marrow taken from control patients.---[128,129]. LOX is a copper-dependent ECM modifying enzyme that functions by crosslinking collagen and elastin precursors, to increase overall ECM stiffness[130]. Under pathological conditions, its dysregulated production coupled with the increased production of fibrillar collagens augments tissue stiffness within the bone marrow niche[131–133]. Although the relationship between the degree of fibrosis and disease prognosis is still unclear; the existence of reticulin fibres alone, indicative of mild fibrosis, do not correlate with disease severity, in contrast, the presence of collagen fibres generated by overactivated myofibroblasts, is associated with abnormal blood count and severity of the disease[134,135]. These myofibroblasts display stronger actomyosin contractility and respond to numerous factors and cytokines secreted by stromal cells, including IL6, TNF-α and TGF-β, by secreting ECM proteins that increase the tissue's stiffness[127]. ECM stiffening will change the physiological conditions of the niche and lead to altered cues received by cells, a process which will eventually compromise haematopoiesis, affect blood cell production and function, leading to a decrease in overall patient survival[102,136].

The impact of fibrosis on tissue mechanics has been well-researched in easier to access tissues such as the lung, liver, and breast [125] . The elastic modulus of healthy lung tissue, typically close to 2kPa, can rise to approximately 17kPa after a fibrotic event[137,138]. Similarly, in liver disease, fibrosis can lead to a significant increase, up to 10 times, in the shear modulus compared to the stiffness of a healthy liver[139]. However, using UV-crosslinked hydrogels based on native porcine digested lung ECM with and without ruthenium (II) chloride crosslinking, =, researchers found that the addition of ruthenium significantly increased the stiffness of the hydrogels by 10-fold which they used as their fibrotic-like condition. They alsofound significantly different $\tau_{1/2}$ stress relaxation times between their control and fibrotic-like hydrogels (1.4s and 1.7s, respectively) that demonstrated that fibrotic conditions may not only increase matrix stiffness but also decrease stress relaxation [140]. Furthermore, compared to controls the fibrotic-like hydrogels demonstrated different fibre characteristics such as increased fibre density and decreased fibre alignment.[140] Finally, increased hydrogel stiffness enhancedmyofibroblast contractility as evidenced by the increased expression of alpha smooth muscle actin and increased matrix deposition.,These processes synergistically increase ECM stiffness, establishing a positive feedback loop.

Similar effects have been found in the breast tumour microenvironment. In vitro work on healthy and tumour tissue taken from murine mammary glands has shown that as breast tumours progressed, the tumour microenvironment exhibited higher matrix stiffness and decreased stress relaxation[141] as well as differences in collagen density and fibre alignment[142]. Additionally, increased collagen density-induced matrix stiffness (from 25 to 44 kPa) in the tumour microenvironment promoted an invasive epithelial phenotype, with increased formation of activated adhesionscoupled with the activation ofFAK–Rho–ERK mechanotransduction signalling network[143]. Therefore, changes in the mechanical microenvironment, caused by fibrosis and/or disease, can induce changes in cellular phenotype that can further dysregulate the matrix mechanics; ultimately potentiating the disease1 [144–146]. However, studies investigating the role of disease-driven mechanical alterations on cell behaviour and differentiation of bone marrow cells, such as mesenchymal stromal cells, have primarily used either 2D systems, that do not recapitulate the tissue's dimensionality, or modelling with primarily elastic materials, that neglect the viscoelastic nature of ECMs in vivo[114]. As a result, a gap still exists when trying to understand the role of viscoelasticity in the bone marrow microenvironment during health and disease.

The time dependent properties of the matrix have been observed in the context of bone marrow myelofibrosis by using interpenetrating networks of alginate and collagen type I fibres which allowed stiffness and stress relaxation to be manipulated independently. Alginate was chosen here due to its inert nature to cells while collagen type I is the most abundant ECM protein in the bone marrow

microenvironment. Human monocyte differentiation was modulated by matrix viscoelasticity as monocytes cultured in stiff slow-relaxing, more elastic substrates differentiated into dendritic cells while those in fast-relaxing, more viscous hydrogels remained as immature monocytes. Transcriptomic analysis further revealed that monocytes in stiff slow-relaxing ECMs expressed cytokines of inflammatory nature, similar to those seen in patients with fibrotic diseases such as myelofibrosis, idiopathic pulmonary fibrosis and liver cirrhosis. In contrast, cells in stiff fast-relaxing gels expressed genes associated with immature myeloid cells, suggesting that tissue stiffening and a loss of viscosity during disease can physically promote a proinflammatory environment within the bone marrow through the selective differentiation of immune cells[98].

The dysregulated ECM remodelling seen during haematological malignancies is characterised by excessive matrix deposition and crosslinking activity as well as disoriented cytokine and chemokine secretion. (Figure 3). Biomechanical and biochemical alterations in the microenvironment create a positive feedback loop to malignant cells and disease progression. Given that the environment exhibits both elastic and dissipative characteristics, it is critical to determine how alterations in the matrix's dissipative properties influence cell function, which cells are most susceptible to viscoelastic alterations and the underlying mechanisms. Additionally, identifying the key changes in stiffness and viscoelasticity can progress the development of ECM-related mechanical markers for early-stage prognosis, as well as therapeutics to restore mechanical properties to healthy-like conditions. To achieve these objectives, bone marrow bioengineered models that faithfully represent the tissue's viscoelastic properties under physiological and pathological conditions must be developed.

[H1]Modelling the bone marrow niche

Engineering humanised bone marrow models that can accurately recapitulate aspects of health and disease have the potential to advance basic bone marrow research and therapeutic development. However, the complexity of the bone marrow niche, characterized by spatially distinct anatomy, variable cellular interactions, ECM matrix chemical and mechanical inhomogeneities and a high degree of vascularization, poses difficulties for faithfully modelling physiological and pathological conditions. 3D models have employed biomaterials, 3D printing, and on-chip approaches to achieve intricate cell-cell and cell-ECM interactions(Figure 4) (Table 1)[147]. Achieving a holistic understanding of the bone marrow environment, including ECM interactions with cells and the incorporation of physical and mechanical characteristics will require a complex combination of approaches.

[H2] Co-culture

Initial in vitro studies of the bone marrow involved culturing naïve hematopoietic progenitors in biomimetic scaffolds with the aim of increasing cell proliferation for clinical needs and drug testing[148]. These studies demonstrated the importance of cellular diversity in bone marrow models as cultures of HSCs alone lose their stemness[149]. Similarly, co-cultures of HSCs with stromal cells such as mesenchymal stromal cells, endothelial cells or osteoblasts revealed the vital role of stromal cell-HSC interactions in governing HSC stemness, growth, maintenance and differentiation[149–154]. For example, when encapsulated in alginate and gelatin-based 3D printed scaffolds, mesenchymal stromal cells as well as the presence of a 3D matrix were able to maintain the stemness of CD34+ haematopoietic stem cells and promote their proliferation compared to 2D controls[150]. Additionally, co-culture of osteoblasts expressing physiologically relevant levels of CXCL12 with human HSCs in an on-chip model was responsible for maintaining HSC stemness and decreasing proliferation. The presence of an endosteal-like niche was also able to prevent HSC apoptosis upon exposure to radiation, suggesting the potential radio-protective role of` osteoblastic cells within the niche[154]. Furthermore, cell-ECM interactions have also been studied using polyethylene-based hydrogels functionalised [155]with 20 bone marrow-related, cell-instructive peptides to recreate the environment's stiffness and enhanced mesenchymal stromal cell proliferation compared to commonly used hydrogels functionalised with arginylglycylaspartic acid (RGD), a cell recognition and attachment site.[155]. Cell-cell and cell-ECM interactions are as equally important for the maintenance and proliferation of cells within the niche and models must consider both of these aspects to balance HSC self-renewal and differentiation.

[H2] Microarray platforms

Microarray platforms are high-throughput systems designed to measure the expression levels of genes or protein sequences. They can be distinguished based on their solid-surface support (e.g. glass, silico chips or nylon membranes), nature of the probe, and the method of probe detection[156]. Biofunctionalized microarray platforms have been developed to investigate mesenchymal stromal cell responses to microenvironment stiffness[157], HSC responses to secreted and ECM-tethered proteins[158] and to identify key HSC-niche interaction ligands[159]. These systems enable high-throughput screening of niche effectors and aim to unravel the multifactorial parameters regulating the bone marrow niche, including biochemical signals from neighbouring cells, soluble molecules and ECM components. For example, a microcavity array was developed to examine the effects of spatial constraint and mechanical properties of embedded hydrogels on HSC fate decisions. The influence of autocrine and paracrine signalling on HSC fate was examined by measuring levels of secreted factors produced at the single-cell level and incorporating these data into a mechanistic machine learning-based model alongside the environmental parameters. The model predicted autocrine signals from HSCs as the main driver of quiescence[160], with the heparin content of the hydrogel exerting the most

microenvironmental influence. This finding underscores heparin's role in binding soluble factors, highlighting the fundamental role of the hydrogel and the active function of the ECM in the soluble niche.

[H2] **Hydrogels**

Hydrogels are soft materials formed by crosslinking hydrophilic polymers. Their water absorbing capabilities allows them to closely resemble the hydrated, soft and viscoelastic nature of the native ECM while their tuneable nature allows precise control over their physical and mechanical properties. In vitro hydrogel systems have been used to investigate paracrine signalling and the biophysical properties of the microenvironment. For example, a methacrylamide-functionalized gelatin hydrogel system that supported mesenchymal stromal cell-mediated remodelling demonstrated dynamic shifts in the ECM biotransport properties after just 24 hours?overtime; when HSC- mesenchymal stromal cells were co-cultured, hydrogels with a small mesh size (~32 nm) and low diffusive properties support mostly autocrine signalling, whereas as mesh size increases (72 nm), paracrine signalling begins to dominate cell-cell regulation[161]. In another study, collagen type I based hydrogels of similar stiffness (~100 Pa) to native bone marrow were used to drive nestin expression in niche stromal cells, and demonstrated an ability to support clinically valuable long-term repopulating HSCs ex vivo . Demonstrating a direct link between the physical, elastic, properties of the ECM in maintaining niche phenotypes[162]. These simplified in vitro systems enable the fine tuning of cellular and microenvironmental properties to provide fundamental insights for understanding the biophysical properties of the niche. Ultimately, this could inform approaches to target the niche as its biophysical properties change throughout ageing and disease processes[163].

[H2] Humanized in vitro models

During haematological malignancies the bone marrow is overflooded with cancerous cells that occupy and transform the niche to support their own survival. To reliably investigate the origin and progression of bone marrow malignancies and the associated molecular pathways (Box 3) In vitro models have focused on representing cell-cell and cell-matrix interactions during malignancy as well as the relationship between LSCs and soluble factors within the environment. For example, the role of the vascular niche in AML was investigated using a 3D PEG-heparin hydrogel in which AML cells were encapsulated along with mesenchymal stromal cells and human umbilical vein endothelial cells[164] . The tri-culture system modelled leukemia-vascular interactions and confirmed that endothelial cells from the vasculature support LSC resistance to chemotherapeutic drugs[164]. Similarly, a microfluidic platform with an encapsulated 3D collagen I matrix demonstrated that LSCs from acute lymphoblastic leukaemia interact directly with osteoblasts and mesenchymal stromal cells in the environment and

that this interaction confers a protective role against chemotherapeutic agents[165]. The role of stromal cells in the leukemic niche was also investigated. Using a hydroxyapatite and collagen I scaffold to model AML, researchers found that upon exposure to human primary AML cells, mesenchymal stromal cells derived from the same AML patient reprogrammed their transcriptome, inducing aberrant cell proliferation and compromised immunomodulatory capacity. To confirm that leukemia converts MSCs into AML-inducing cells, healthy human mesenchymal stromal cells were cultured with AML leukemia blasts. This resulted in a dynamic shift in their transcriptome and secretory profile so that they resembled those of the AML-derived stomal cells. This model was able to show that cancer cells functionally alter the resident stromal cell population to ensure progression of the disease and conversion of the microenvironment towards one that supports cancer cell survival[166].

[H2] Modelling matrix mechanics

These studies have demonstrated the roles stromal cells play in HSC and LSC fate and have attempted to reproduce certain spatial aspects of the bone marrow niche, however they still lack biomechanical relevance. Studies have highlighted the fundamental role of matrix stiffness on the successful functioning and reconstitution of the healthy bone marrow niche ex vivo by using hydrogels of different stiffness to investigate HSC lineage specification---[88]. Even though remodelling of the niche is now a well-established hallmark of such diseases, the effect of haematological malignancy on matrix mechanics and vice versa is not completely understood. Recently, alginate hydrogels were used to show that matrix stiffness regulates AML cell proliferation, tumour growth and chemosensitivity. Specifically, increased matrix stiffness (3kPa compared to 0.1kPa) resulted in a biphasic proliferation response for AML cells but not CMLcells, whose proliferation decreased, suggesting that, while mechanical cues are important for disease development, they may differentially regulate cell proliferation in the context of different leukaemia subtypes. Additionally, after implanting alginatehydrogels in an in vivo mouse modelof extramedullary myeloid leukemia, researchers confirmed that tumour growth and chemotherapeutic response can be regulated by matrix stiffness[167]. Specifically, when soft (0.1 kPa) and stiff (3.0 kPa) hydrogel discs encapsulated with K-562 myeloid leukemic cells were added to the mice, researchers observed that increased stiffness decreased the initial growth rate as well as the deceleration rate by 1.5-fold and made leukemic cells more sensitive to chemotherapeutic agents.

The matrix's time-dependent properties influence cell functions and disease progression in the malignant bone marrow microenvironment. Their importance in malignancy has been demonstrated in studies on liver and breast cancer[168,169]. For example, advanced glycation end-products,produced in type-2 diabetes, were investigated in the context of hepatocellular carcinoma. These were found

tocause structural changes to the ECM increasing liver viscoelasticity.. A faster stress-relaxing niche can enable activation of mechano-signalling that encourages hepatocellular cancer progression, independent of stiffness[169]. Furthermore, in breast cancer, matrix plasticity modelled in vitro by highly viscous nanoporous networks of alginate and reconstituted basement membrane matrix was found to be an important regulator of human breast cancer cell invasion and metastasis irrespective of stiffness-related cues[170]. The effect of viscoelasticity during haematological malignancies has been explored in a 3D bioengineered alginate/collagen type I hydrogel model of primary myelofibrosis. The authors found that both matrix stiffness and viscoelasticity can independently control monocyte differentiation in the fibrotic bone marrow niche[98]. Specifically, this artificial ECM system demonstrated that human monocytes cultured on stiff elastic hydrogels showed proinflammatory polarization and differentiation towards a dendritic cell lineage compared to those cultured on viscoelastic matrices that maintained an immature monocyte profile. However, a major gap still exists in understanding how viscoelasticity affects healthy HSCs, stromal cells or LSCs and how these biomechanical cues can be used in modelling to increase *in vivo* relevance. Addressing this gap will require the development of accurate, controllable viscoelastic hydrogel platforms that can be used to decouple the effect of different ECM mechanical cues on hematopoietic, stromal and leukemic cell function. Advanced models incorporating spatiotemporal mechanical cues can improve in vivo relevance, enabling accurate modelling and better insight into mechanical-specific disease mechanisms.

[H1]Outlook

The bone marrow is a mechanically dynamic tissue. The heterogeneity of the ECM in the bone marrow microenvironment provides biochemical, biomechanical and biophysical stimuli to maintaining the delicate balance of HSC homeostasis. Haematological malignancies cause rearrangement of the ECM and alterations in the microenvironment's mechanical properties that are detrimental to healthy cells while supporting the survival of malignant cells[102]. Yet despite the role of tissue mechanics in the bone marrow under both physiological and pathological conditions, our understanding remains limited due to the inaccessibility of this tissue *in situ*. Bioengineered models offer an avenue to address this, by leveraging insights from mouse models to inform design of *in vitro* platforms that can accurately recapitulate the native mechanical properties of the tissue. Engineering of systems with reliable stiffness and stress relaxation or creep profiles tailored to the specific sub-niche of interest are already in development[89,98]. Ideally, matrices should mimic the native ECM's weak and covalent bond structure to incorporate the observed biofunctionality of the *in vivo* microenvironment. Systems utilizing interpenetrating or double network-based matrices, incorporating both covalent and ionic crosslinks, present promising platforms for exploring how these bonds contribute to the ECM'S time-dependent

properties particularly when compared to matrices that are exclusively ionically or covalently crosslinked. Such “hybrid” systems could serve as more accurate models for studying the influence of matrix mechanics on cell behaviour under physiological and different pathological conditions. [98,99]. Additionally, consideration of the role of cellular diversity within the bone marrow will be essential for developing informative models.

Replicating the mechanical and cellular complexity of the bone marrow microenvironment remains a challenge. Development of mathematical and computational modelling approaches would provide insights into the effects of tuning ECM mechanics to produce desired cell function, and the impact on cell responses in malignancy. Advanced imaging techniques such as Brillouin and Raman spectroscopy, magnetic resonance elastography or multiplexed tissue imaging with co-detection by indexing (CODEX)[171] hold promise for furthering our understanding of the in situ bone marrow niche environment. Such tools are equipped with high spatial resolution and have the potential to inform and enhance the design of in vitro bioengineered models. Their predictive and non-invasive nature also aids the replacement, reduction and refinement (3Rs) of animals needed in research, and improves our understanding of complex biological systems.

Ultimately, understanding the role of the physical microenvironment in maintaining homeostasis and driving pathogenesis is key for advancing future treatments of bone marrow diseases. Bioengineering models that accurately mimic bone marrow matrix mechanics and can be used with patient-specific cells can aid this effort, paving the way for the development of novel personalised therapeutic strategies, including mechanical biomarkers of disease progression, and increase the confidence in bioengineered models as preclinical testing platforms. In this review we have highlighted how matrix mechanics, such as stiffness and viscoelasticity, can influence immune cell activation, direct stem cell lineage specification and contribute to disease progression by promoting cancer cell proliferation & migration. It is therefore possible to develop novel therapeutics that target matrix mechanics with the aim to control immune responses, guide stem cell behaviour and secretome, and restore tumour stiffness in a personalised manner tailored to individual patient needs. Targeting tissue mechanics could also be used combinatorically, to increase infiltration of more traditional drugs and immune cells, improving immunotherapy efficacy in solid tumours. Ultimately, integrating these mechanically driven approaches with existing treatments might prove more effective in addressing both the biochemical and biophysical aspects of bone marrow diseases.

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This study uses ECM mimics and hydrogels to recreate the low-stiffness bone marrow microenvironment, demonstrating that this drives nestin expression in stromal cells, which in turn support clinically valuable HSCs.

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This study shows that biophysical cues of the matrix such as its stiffness can modulate leukemic cell proliferation and drug sensitivity.

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Competing Interests

The authors state no competing interests.

Conflict of Interest

The authors declare no conflicts of interest.

Key points

- The bone marrow niche is a complex tissue, but its mechanics are not well understood owing to the challenges of studying it in situ..
- In haematological malignancies, cancer cells remodel the microenvironment to support their survival and metastasis. This remodelling is characterised by a dysregulated biochemical milieu and altered matrix deposition that changes the mechanical properties of the bone marrow.
- Bioengineered models that mimic the bone marrow must faithfully represent the ECM mechanical properties, such as viscoelasticity, in health and disease.

Display items

Table.7¡Bioengineered.models.investigating.mechanical.properties.of.healthy.and.diseased.bone.marrow¡.

Condition	System	Materials	Cells	Mechanical properties	Investigation of viscoelasticity	Remarks	Ref
Healthy	3D hydrogel	PEG-heparin	Human CD34+ cells	*G'* = 0.5-3 kPa	No	Maintenance of HSCs through the independent adjustment of biophysical and biochemical cues.	89
	3D hydrogel	GelMa	Coculture of murine HSCs and MSCs	*G'* = 0.2-10 kPa	No	Matrix stiffness negatively regulates bone marrow stem cell niche factor expression as well as reconstitution of the bone marrow niche ex vivo.	88
	3D bioprinted hydrogel	Methylcellulose / alginate	Coculture of MSCs and ECs	*G'* = 0.1-1 kPa	No *Viscosity of bioink investigated	Hydrogels sustained MSCs and ECs for 7 days. Different stiffness regimes were optimal for each stomal cell type.	172
	3D hydrogel	Alginate	Murine HSPCs	*E'* = 20-70 kPa	No	Hydrogels with larger pore size and higher stiffness favours HSPC proliferation and maintenance of stemness.	87
	3D hydrogel	GelMa	Murine HSCs & MSCs	*E'* = 1.5-4 kPa	No	Functionalisation of hydrogels to support HSC viability during in situ gelation and inclusion of peptide crosslinker to reduce surface marker degradation for post-culture analysis.	173
	2D polymer & 3D hydrogel	FN coated Poly (ethyl acrylate) coverslip and collagen type I hydrogel	Human perivascular stem cells & HSCs	*G'* = 0.08 kPa	No	Low-stiffness hydrogels drive a niche-like phenotype in stromal cells, that can support functional long-term HSCs.	162
Diseased	3D hydrogel	IPN of alginate and collagen type I fibres	Human monocytes	*E'* = 7.5 kPa	Yes	Viscoelasticity contributes to the aberrant differentiation of monocytes in the fibrotic bone marrow niche.	98
	3D hydrogel	Alginate	AML cells (MOLM-14, U-937) or CML cells (K-562)	*E'* = 0-3 kPa	No	Matrix stiffness regulates AML cell proliferation and chemosensitivity to drugs.	167

	3D hydrogel in microfluidic system	Collagen I	Human ALL cells (SUP-B15), BMSCs & osteoblasts	G' = 0.3 - 1.2 kPa	No	ALL cells in the 3D tri-culture system exhibited decreased chemotherapeutic drug sensitivity. System was able to also demonstrate cell-cell, cell-matrix interactions.	[165]

HSC; Hematopoietic stem cells, MSC; Mesenchymal stem cell; EC; Endothelial cells, HSPC; Hematopoietic stem and progenitor cells, FN; Fibronectin, IPN; Interpenetrating networks, AML; Acute myeloid leukemia, CML; Chronic myeloid leukemia, ALL; Acute lympoblastic leukemia, BMSCs; Bone marrow mesenchymal stem cells.

Figure 1 | The central bone marrow of the femur is a primary site of haematopoiesis and haematological malignancies in humans. (a) Vertical cross-sectional view of an adult human femur shows the arrangement bone layers lining the central bone marrow. The periosteum lines the outer surface of the bone while the endosteum is located at the interface of the bone marrow and bone. Sympathetic nerves line the arterioles and innervate the bone marrow niche, providing essential cues for HSC migration in and out of the niche[200] . (b) Within the bone marrow, only long-term HSCs (LT-HSCs) reside close to the endosteum and self-renew and differentiate in to short-term HSCs (ST-HSCs)to repopulate the bone marrow throughout a lifetime. Short-term HSCs (ST-HSCs), then migrate close to vessels and can further differentiate into haematopoietic progenitor cells of the myeloid or lymphoid lineage to produce all the blood and immune cells of the body.

Figure 2 | Schematic representation of the healthy HSC niche in the adult bone marrow. The bone marrow is separated into distinct subniches characterised by different cell types that secrete niche factors to directly or indirectly regulate HSC activity and haematopoiesis. The variable protein distribution gives rise to different mechanical properties within the bone marrow, which also influence HSC and stromal cell fate and function. Oxygen concentration also varies within the bone marrow microenvironment with the endosteal niche being slightly more oxygenated than the central bone marrow. Ang-1, Angiopoietin 1; SCF, stromal cell factor; TPO, thrombopoietin TGFβ1, transforming growth factor beta 1; VEGF, vascular endothelial growth factor.

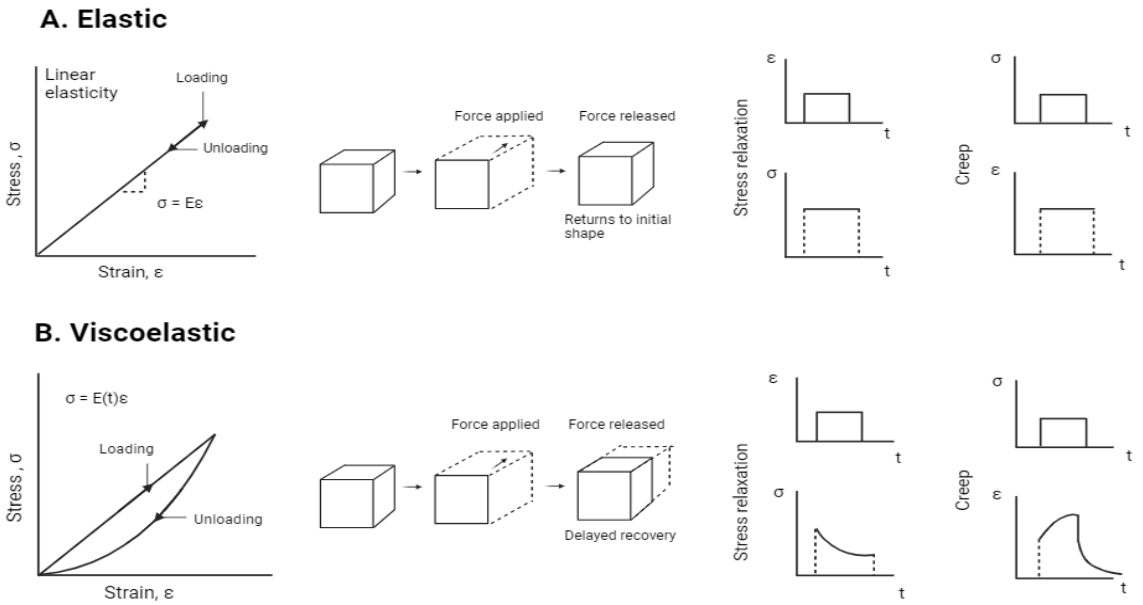
Figure 3 | Malignancy-induced micro-environmental changes in the bone marrow niche. (a) Biochemical factors such as CXC motif chemokine 12 and Bone morphogenetic protein 2/4 produced by niche stromal cells increase malignant cell survival, homing, and expansion in the niche. (b) Once engrafted, remodelling of the niche occurs as the production of pro-inflammatory cytokines by malignant cells that stimulate other cells to express anti-apoptotic factors and by the production of proinflammatory cytokines by immune cells, both of which create an overall change in the biochemical milieu in the niche. (c) The niche's pro-inflammatory profile also stimulates malignant and other stromal cells, such as fibroblasts, to secrete excessive amounts of collagen I, reticulin fibres and enzymes with crosslinking activity, that effectively alter the mechanical properties of the bone marrow's ECM.

Figure 4 | Overview of different approaches used to model the healthy and diseased bone marrow niche. From (a) to (c) bioengineered models are ordered with regards to their structural and dimensional complexity. On the right examples and uses of (a) 2D approaches (b) 3D model approaches and (c) organ-on-chip approaches are provided [147,174]

Box 1 | Tissue viscoelasticity in health and disease

Many biological tissues, such as the brain, liver, kidney, spleen, pancreas, adipose and myocardial tissue, bone, cartilage and tendon [90]exhibit elastic and viscous characteristics under deformation, a property known as viscoelasticity. Viscoelasticity allows tissues to transition from an initial elastic response akin to elastic solids, to a time-dependent response when subjected to mechanical loading. As such, viscoelastic solid materials, including tissues, exhibit stress relaxation in response to a constant strain and exhibit creep in response to a constant stress meaning ----. They also dissipate some of the energy it took to deform them, usually in the form of heat[91,92]. However, unlike viscoelastic

liquids that continuously dissipate an applied strain, viscoelastic solids will eventually store the applied strain and the measured stress reaches a plateau. The viscoelastic nature of materials is quantified by measuring stress relaxation, creep and the loss tangent (tanδ). Stress relaxation is the time it takes for stress to relax to half its initial value ($\tau_{1/2}$), creep is the time it takes for strain to increase over time, and the loss tangent is the ratio between G' and G'' which indicates the dissipative nature of a material. This is highly frequency-dependent, with G' and G'' increasing with the frequency of the stress/strain.



Schematic of (a) elastic and (b) viscoelastic with respective stress-strain curves (left), stress relaxation and creep behaviours (right).

Rheology used to assess the bulk viscoelastic properties of materials as stresses and strains can be applied in shear or axial directions. Atomic force microscopy and particle-based microrheology can assess viscoelasticity at a microscale level[92,94]. A combination of these techniques have been used to measure the viscoelastic properties of tissues and reconstituted extracellular matrices (ECMs)[85,86,95].

Cells interact with the ECM by exerting forces through integrin-based adhesions[175]. This enables cells to sense and convert the ECM's mechanical cues into biochemical signals –known as mechanotransduction[175–177]. Therefore, the ECM's time-dependent properties affect mechanostransduction and cell behaviour. For example, increasing stress relaxation in—hydrogels?---promotes cell spreading,[178,179] and regulates differentiation processes[99,180] independently to the initial elastic modulus. Changes in ECM mechanics can also influence disease progression or treatment. In vitro experiments using highly viscous nanoporous networks formed by alginate and reconstituted basement membrane matrix, demonstrated breast cancer cells migrate through these matrices in a protease-independent manner through invadopodial protrusions[170]. This suggests that viscoelasticity

governs cell confinement and movement in a diseased state, and is therefore an important factor to be considered when studying disease progression and metastasis[103].

Box 2 | Measuring bone marrow mechanics

Research on bone marrow mechanics is challenging, primarily because of the inaccessibility of the tissue. Additionally, the physical, biochemical and cellular composition of the bone marrow are closely linked to its mechanical properties[8] and structural changes caused by physical handling, dehydration or swelling as well as temperature variations upon removal can alter the tissue's intrinsic properties. Blood coagulation within the bone marrow poses another challenge as exposure to air can cause blood cells and plasma located within the bone marrow to coagulate, changing the tissue's mechanical features. Although the use of anti-coagulants can mitigate these effects, accurately assessing bone marrow mechanics without disrupting its natural environment and intrinsic properties remains difficult[97] .

Bone marrow mechanics have been measured by homogenising the tissue and then using traditional macroscopic methods such as rheology to assess the tissue's bulk mechanical properties[181]. However, this approach is destructive and lacks physiological relevance. Techniques such as atomic force microscopy and cavitation rheology, alongside traditional rheology, offer a way to measure intact bone marrow from animals such as bovine, porcine and mouse [86,97,182]. Atomic force microscopy measures the forces between a probe and sample, and cavitation rheology investigates the mechanical properties of a tissue through the formation of cavities within the tissue; both can aid in obtaining data that reflect the local mechanical characteristics of a tissue, at scales relevant to cellular interactions (µm scale). Similar to rheology, these have shown that the mechanical properties of the bone marrow differ depending on the location of measurement. However, differences in the scale of measurement and tissue sources have made comparison of results difficult between studies and new methods that will allow quantification of bone marrow mechanics in situ are needed.

Advanced imaging techniques coupled with mechanical testing such as ultrasound elastography and magnetic resonance elastography offer non-invasive methods for imaging deep within the body and provide quantitative data on tissue mechanics[183]. Ultrasound elastography uses high-frequency waves to generate tissue images[184], and magnetic resonance elastography employs magnetic resonance imaging to visualise mechanical wave propagation within tissues[183,185]. Both techniques offer potential as clinical tools for the assessment of different diseases including fibrosis, cancer, inflammatory bowel disease, cardiovascular disease and hyperplasia. For example, ultrasound elastography and magnetic resonance elastography can be used to evaluate different stages of liver fibrosis in a non-invasive

manner[183]. Although these techniques come with challenges including technical sensitivity, operator dependency as well as tissue and patient-depending factors (e.g. obesity) that might complicate clarity of imaging --- they could be possible improved by incorporating machine learning algorithms that can correct artefacts in real time and decrease errors due to operator dependency. These improvements could enable a more standardised and reliable quantification of mechanics in difficult to access tissues, such as the bone marrow, by minimising structural alterations and preserving cellular interactions and the native microenvironment.

BOX 3: Technology transfer of bioengineered bone marrow models.

Around 1 million animals are used each year in the United Kingdom alone for industrial Pharma and toxicology screening (gov.uk) however the biological mismatch between animals and humans means they do not accurately predict clinical outcomes. Despite unprecedented levels of investment in pharmaceutical research and development[186], most drugs are failing in the clinical stages[187]; for example, 95% of cancer therapeutics that enter phase I of clinical development fail to gain market authorisation[188] . Because animal models fail to convert preclinical findings into meaningful clinical outcomes, approaches using bioengineered in vitro human organ models are gaining precedence. Both funders and Pharma have produced roadmaps for the development, validation and adoption of in vitro models, and regulators in the United Kingdom and United States are working toward legislative changes to allow inclusion of in vitro human organ model data instead of, or alongside, in vivo data in medicinal product development dossiers. Advancement and application of appropriate models will allow greater predictive power early in therapeutic development by improving the rate of translation and reducing costs and to ultimately benefit patients. Several challenges must be addressed for bioengineered models to gain acceptance by pharmaceutical and regulatory agencies. [bH1] Translation challenges

[b1] Biological relevance

[b2]. Models must be biologically relevant, accurately mimicking the physiological tissue by using human cells and tissues and incorporating key microenvironmental cues. Model type (2D vs 3D), the experimental set up (perfusion vs static) and the desired output must all be considered to source and select appropriate cells and tissues. **[b1] Complexity**

[b2]The model must be as complex as needed for its specific purpose to function and provide the required output. For example, in drug screening applications 2D high throughput methods using patient-derived cells are sufficient to enable time-effective testing of a large compound library. In

contrast, disease modelling applications require, 3D organ models with biological and structural heterogeneity to provide insight into disease mechanisms. **[b1] Functionality**

[b2] Relevant tissue and disease functions should be demonstrated, such as responses to disease-relevant stimuli, or expression of disease-relevant or tissue-relevant biochemical and biomechanical markers.**[b1] Validation and standardization**

[b2]Responses of the models should be compared to known outcomes in pre-validated in vitro assays, and to known outcomes in animal and clinical human data. Once models are validated, standardized protocols for their generation and quality control must be established to enable application across different laboratories.

[b1] Regulatory acceptance

[b2] regulatory requirements for safety and efficacy assessment must be outlined and met to replace and reduce the number of animals used in preclinical testing. Cross-sector engagement between researchers and regulatory bodies is essential.

Short Summary

In this Review, we demonstrate the importance of understanding the complex mechanical behaviour of the bone marrow niche, how this changes with disease, and how bioengineered models can be used to uncover, replicate and target physiological and pathological mechanical signatures of the bone marrow.