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Advances in nanoscopic mechanobiological structure-property relationship in human bones for tailored fragility prevention

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Abstract

It is well documented that fragility fractures represent an enormous health, economic and psycho-social burden, leading to severe pain, loss of mobility, and even death. While clinical approaches focusing on macro down to micro-scale damage in bones are often ineffective to diagnose early fracture occurrence, nano-scale investigations are opening new frontiers for targeted fragility prevention. This review highlights a novel triad that merges advanced nano-imaging techniques, nano-mechanical characterization and finite element/molecular dynamics-based computational models to elucidate the structure-property relationship that leads to bone fractures. Techniques such as atomic force microscopy and high-resolution electron microscopy enable the evaluation of mechanobiological mechanisms and damage occurrence at the sub-micro scale, providing visualization of bone ultrastructure. Simultaneously, nanoindentation and micropillar compression offer precise measurements of mechanical properties, unraveling how bone responds to diverse forces. Pertaining computational tools, nano-scale modeling simulations explore the behavior of bone components under varying conditions, yielding crucial insights into fracture mechanisms. This holistic triad unveils interactions between mineralized collagen fibrils, cross-links, and bone structures, leading to targeted prevention and personalized treatment of bone fragility, by addressing their root causes at the nano-scale, potentially lowering their incidence and severity.

Keywords

Fragility fractures; nano-scale imaging; nano-mechanical testing; nano-scale modeling

1. Introduction

Bone fragility fractures pose a significant global health challenge with profound implications for individuals and society at large, encompassing health, social, and economic dimensions [1]. The surge in bone fragility fractures is intimately tied to the substantial loss of mineral density and alterations in the multi-scale architecture of bone tissue. Osteoporosis, a systemic disease of the skeletal system, plays a core role in elevating the risk of fractures, positioning it as a critical health concern for the elderly, second only to cardiovascular disease, according to the World Health Organization (WHO) [2].

Disparities in fracture rates across different countries, notably higher in North America and Europe, underscore the urgent need for comprehensive strategies [3]. It is alarming that an estimated 75 million people in Europe, the USA, and Japan are affected by osteoporosis, resulting in over 2.3 million fragility fractures annually in Europe and the USA alone [4]. The global toll of fragility fractures reaches approximately nine million accidents each year [5]; this means that the societal burden of fragility fractures is dramatically impactful and extends beyond individual suffering to encompass substantial direct medical costs. The annual direct cost of treating fragility fractures in Canada, Europe, and the USA alone is estimated to range from 5000 to 6500 billion USD [4]. Moreover, osteoporosis-related fractures impose a considerable impact on healthcare resource utilization and costs, extending beyond the direct expenses of acute fracture treatment [6]. Besides, as the average age of the population increases and lifestyle habits evolve, the incidence of fragility fractures is projected to rise significantly in the future, up to 4.5 million cases by 2025 in Europe alone [5]. Recent statistics [1,7] also highlight regional disparities and the growing impact of osteoporosis

and fragility fractures globally [8]: for instance, in North America and Europe, the fracture rates are notably higher compared to other regions, with rates as high as 1 in 3 women and 1 in 5 men over the age of 50 experiencing osteoporotic fractures [9]. In the United States, approximately 54 million adults aged 50 and older are affected by osteoporosis and low bone mass, contributing to an estimated 2 million fractures annually [9]. Similarly, in Europe, the economic burden of osteoporosis and associated fractures is substantial, with direct costs of treatment amounting to over 37 billion euros annually [9].

Elderly individuals, particularly women aged 65 and above, face an increased risk, specifically due to hormonal changes during menopause and smaller bone size. Additionally, women's longer life expectancy results in greater reductions in bone mass over their lifespan. The consequences of fragility fractures among the elderly are severe, encompassing hospitalization, long-term care, impaired quality of life, and disability. The incidence of fractures rises exponentially with age due to increased skeletal fragility characterized by low bone mass and deficits in bone geometry, microarchitecture, and material properties. Additionally, age-related risk factors for fractures include increased falls, vitamin D deficiency, calcium insufficiency, and inadequate protein intake. Vitamin D deficiency, in particular, is linked to muscle weakness, elevated fracture risk, and worsening conditions of osteoporosis [5,10]. Another critical risk factor for future fractures is a history of previous fragility fractures, with individuals experiencing three or more fractures facing a ten-fold higher risk compared to those with no fractures [11]. The most common osteoporosis-associated fractures affect the hip, spine, and forearm. Hip fractures, being the most serious, necessitate hospitalization and result in a significant reduction in independence for 50% of affected women, leading to long-term care in 20% of cases [10]. Vertebral fractures impact patient quality of life, albeit to a lesser extent than hip fractures. Distal forearm fractures, common among middle-aged and elderly individuals, are rarely fatal but may necessitate hospitalization.

The evolving definition of osteoporosis emphasizes a shift from a focus solely on low bone density to a broader consideration of high bone fragility [2,12] . Bone fragility, a composite descriptor of bone multi-scale mechanical properties, directly influences fracture susceptibility and is inversely related to a bone ability to resist fractures [13]. Mechanical properties such as hardness, modulus, and toughness serve as direct quantitative indicators of fragility. However, these mechanical measurements are often impractical in clinical settings, leading to their underestimation by practitioners.

Indeed, understanding fragility fractures requires a comprehensive approach that spans from macroscopic epidemiological trends to microscopic biological mechanisms. While macro-level studies provide insights into population-level risk factors and societal impacts, and micro-level investigations focus on individual bone structure and genetic predispositions, a nano-scale approach is essential for uncovering intricate molecular interactions within bone tissue. Nano-scale studies enable researchers to examine the collagen matrix, mineralization patterns, and cellular interactions at a level that influences fracture initiation and propagation. This detailed understanding not only enhances the knowledge of bone health but also paves the way for targeted therapies and personalized medicine strategies aimed at reducing fracture risk effectively. Thus, integrating nano-scale analyses into fracture research promises to revolutionize how clinicians prevent and manage osteoporosis and related fragility conditions in the next future.

1.1 Background

Clinical prognoses of bone fracture risk rely on an array of techniques, each with distinctive attributes and limitations (Figure 1). However, current medical imaging modalities predominantly focus on the macro- and mesoscale, complicating the identification of damage processes at the nano-level, thereby necessitating higher-resolution methodologies [14].

Dual-energy X-ray absorptiometry (DEXA), acknowledged as the gold standard for assessing bone mineral density, is widely used for estimating osteoporosis risk and monitoring therapeutic efficacy. Nevertheless, DEXA primarily focuses on bone density analysis, sidelining critical determinants of bone quality such as composition, geometry, micro-architecture, and spatial distribution[15]. DEXA's methodological constraints, such as its sensitivity to variations in bone size, undermine bone mineral density as a reliable predictor of fracture risk. [2,14,16,17].

The exigency to address the multifaceted nature of bone health propels a nuanced approach. The FRAX algorithm, combining bone mineral density measurements with clinical risk factors, provides an estimation of the decade-long probability of major osteoporotic fractures. Nonetheless, the inherent limitations of FRAX become apparent, as it fails to include certain pertinent risk factors [10,18].

Three-dimensional quantitative computed tomography (QCT) allows distinguishing cortical and trabecular bone components, providing insights into bone geometry and density, essential in fracture risk assessment [19]. Peripheral quantitative computed tomography (pQCT) enables live 3D imaging of peripheral bone sites, capturing selected geometric parameters associated with bone strength [16,17,19]. High-resolution pQCT (HR-pQCT), additionally, delineates bone microarchitecture parameters at a finer resolution.

Micro-CT, a non-destructive modality, shows 3D bone morphology at a resolution spanning 1-100 µm [12]. Coupled with contrast agents, this technology emerges as a promising approach for quantifying microdamage induced by various pathologies or mechanical loading, thereby facilitating the prediction of mechanical stresses and strains [20,21]. Nevertheless, its limitations lie in the inability to provide insights into cellular functions and remodeling activity, necessitating parallel histological examinations.

Finite element analysis (FEA), grounded in in vivo images from HR-QCT, stands out as a more precise method for estimating bone strength and appraising fragility. Beyond estimating the strength and stiffness of bone tissue, FEA excels in delineating stress distributions, demonstrating a predictive accuracy of fracture positions in a substantial proportion of cases [22,23]. The efficacy of FEA, however, needs high-resolution tomography images to obviate distorted outcomes [12,24].

Magnetic resonance imaging (MRI) introduces an additional dimension by leveraging a magnetic field and radio frequency pulses to generate intricate 3D images [17,25,26]. MRI, including low-field magnetic resonance imaging (LF-MRI), allows elucidating structural alterations due to age and osteoporosis [34]. The synergistic integration of micro-CT and LF-MRI has showcased the potential to discern qualitative alterations in trabecular bone concomitant with mechanical behavior [27]. LF-NMR further augments our understanding by characterizing micro-damage in cortical bone following mechanical loading [28]. Lastly, ultrasound imaging stands out as a non-invasive, cost-effective, and portable technique that provides real-time imaging without radiation exposure, but it has limited tissue penetration, is operator-dependent, and cannot assess bone micro-architecture [29].

Given the escalating prevalence of fragility fractures, a paradigmatic shift toward preemptive strategies becomes urgent: delving into the nano-scale intricacies of bone architecture, unattainable through conventional clinical practices, represents a step forward in fracture prevention and the improvement of patients' quality of life.

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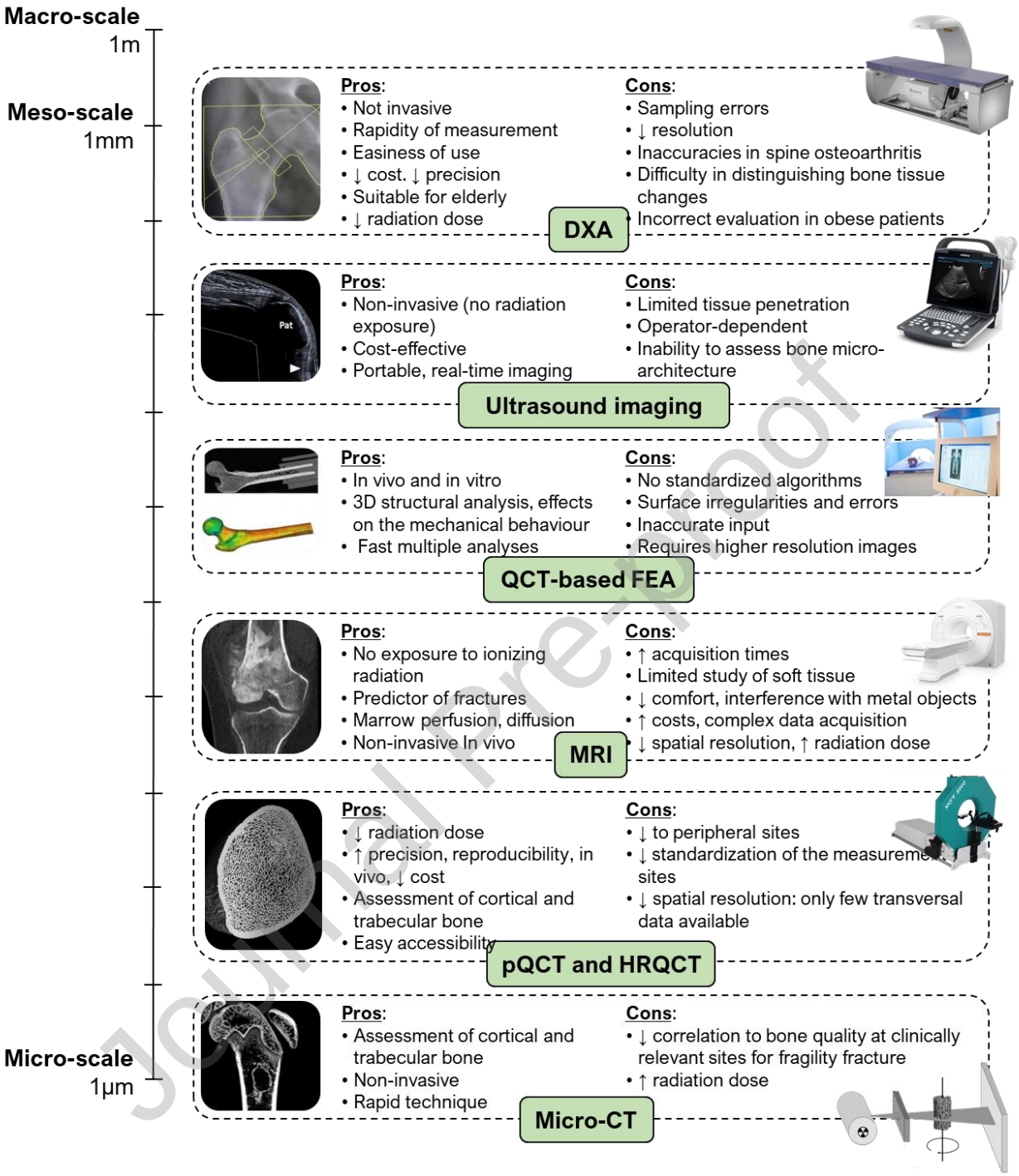

Figure 1| Overview of the main clinical methods to assess bone fragility.

1.2 Current status and challenges

To mitigate the economic and health impacts of fragility fractures globally, there is an imperative need to prioritize preventive care and early diagnosis, delving into how fractures originate at smaller scales, before evolving into critical large scale fractures, that could be detected via conventional diagnostical tools detailed in 1.1. More in depth, understanding and manipulating bone nano-scale mechanobiological mechanisms can provide unprecedented insights into fragility and fracture resistance, offering a pathway towards more effective preventive strategies.

Specifically, the nano-scale architecture of bone, involving structures at dimensions of nanometers, plays an essential role in determining its mechanical integrity. At this level, the interactions between mineralized collagen fibrils, hydroxyapatite crystals, and other nanostructures contribute significantly to the overall strength and resilience of bone. Any disruption or alteration at the nano-scale can have profound consequences on the mechanical properties of bone, influencing fragility and fracture susceptibility.

To shed light on nano-scale mechanobiological mechanisms inducing early fragility signs a combination of ***advanced imaging techniques***, ***nano-mechanical characterization*** and ***finite elements/molecular dynamics-based computational models*** are required. This triad is essential for bridging the gap between the research and the clinics, to elucidate and actively prevent fragility signs (see Figure 2).

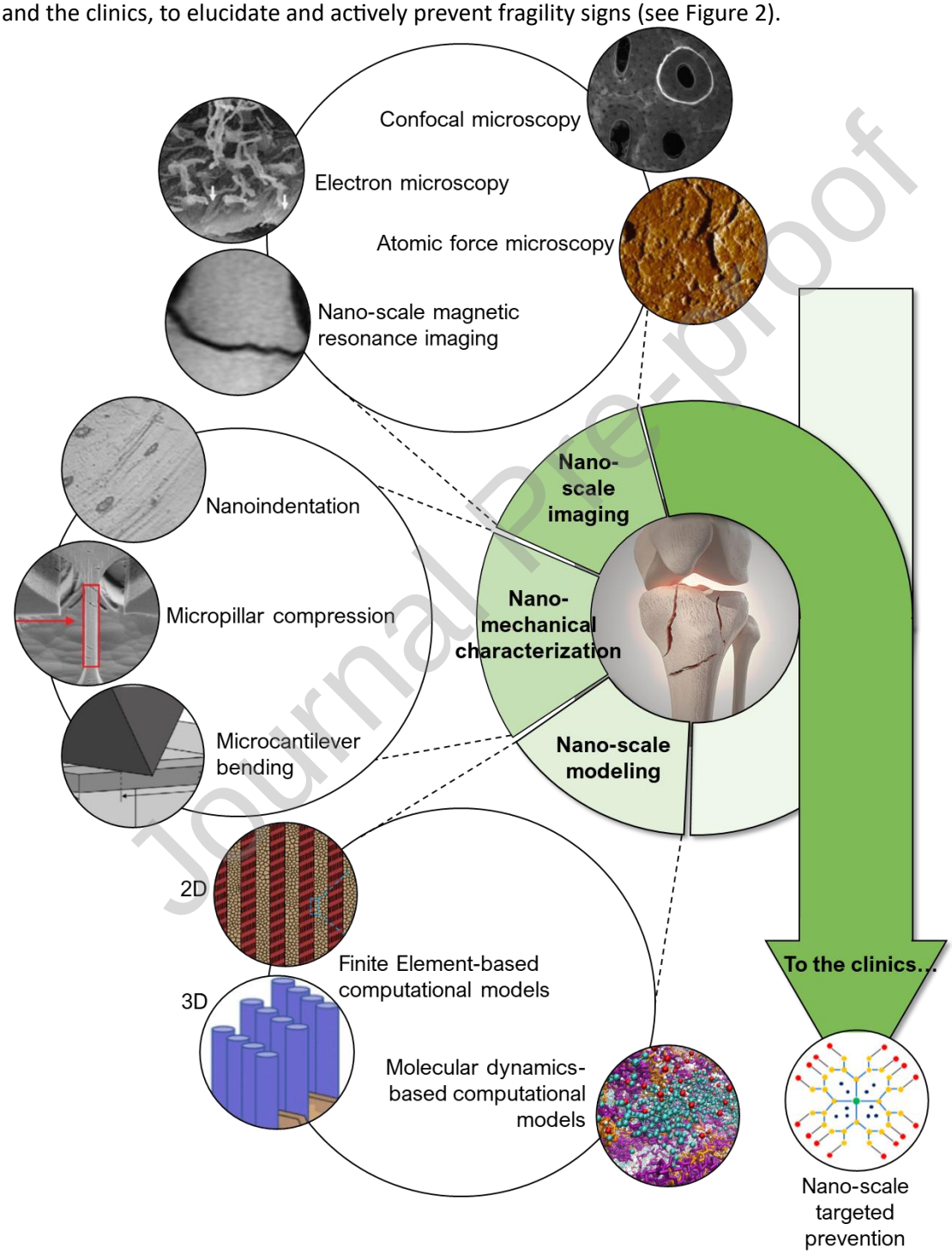

Figure 2| Combination of advanced imaging techniques, nano-mechanical characterization and finite elements/molecular dynamics-based computational models for elucidating nano-scale mechanobiological mechanisms inducing early fragility signs.

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Recent advancements in nanotechnology and materials science present an opportunity to explore and manipulate the nano-architecture of bone for enhanced fracture prevention. By gaining a deeper understanding of the nano-mechanical properties, such as hardness, modulus, and toughness, researchers could develop targeted interventions to reinforce bone tissue at its most fundamental level.

Moreover, advancements in nanomedicine open avenues for innovative diagnostic and therapeutic approaches. Nano-scale imaging techniques allow for precise visualization and characterization of bone structures, aiding in the early detection of nano-level alterations that may precede macroscopic fractures. Targeted drug delivery systems at the nano-scale can also be designed to enhance bone strength and mitigate the progression of osteoporosis, thereby reducing the risk of fragility fractures.

Incorporating nano-scale considerations into clinical assessments and preventive care strategies is crucial for a more holistic approach to bone health. While traditional clinical measurements often focus on bone density and macroscopic architecture, acknowledging and addressing nano-level intricacies can provide a more accurate and nuanced understanding of bone fragility. It is essential for practitioners to embrace emerging technologies and research avenues that explore the nano-scale mechanics of bone, translating these findings into practical applications for fracture prevention.

By bridging the gap between macroscopic and nano-scale perspectives, a more robust foundation for preventive care and early intervention could be stablished. This interdisciplinary approach, combining biomechanics, nanotechnology, and medical science, holds the promise of revolutionizing the ability to safeguard against bone fragility fractures in the face of an aging population and evolving lifestyles.

2. Nano-scale bone structure

2.1 Bone as a living tissue: a multi-scale hierarchical organization

Detecting bone fragility presents a formidable challenge for diagnostic tools due to the intricate hierarchical architecture of bone across various scales. The human skeleton serves multiple mechanical roles, such as providing support, enabling movement, and safeguarding vital organs. Additionally, bone plays a non-mechanical role in maintaining whole-body homeostasis by regulating calcium and other mineral levels, facilitating mineral deposition, supporting hematopoiesis, and absorbing toxic minerals [30–32].

Bone, a mineralized connective tissue, comprises a mineral phase (70% hydroxyapatite) and an organic phase (30% collagen, non-collagenous proteins, bone cells, and water) [33]. The primary orchestrators of continuous bone remodeling are bone cells, overseeing the processes of resorption and deposition [34]. Osteoclasts break down old bone tissue, osteoblasts form new bone tissue, and osteocytes play a central role in remodeling, cellular communication, and supporting daily skeletal functions [35–37]. The balance between bone formation and resorption, influencing mass, morphology, and tissue properties, is a biological process crucial for bone resistance to fractures [38].

Bone tissue exhibits a complex hierarchical organization, necessitating an understanding of its composition and mechanical properties at each level scale to elucidate its overall mechanical characteristics. At the ***macro-structural level*** (50 cm-10mm), bone is considered as part of the skeletal system, with 20% of the skeletal mass made of trabecular tissue and 80% of cortical tissue, each characterized by distinct functions and architecture [31].At the ***meso-structural level*** (10 mm-500 µm), cortical (dense) and trabecular (spongy) bone types are distinguished. Cortical bone forms the hard exterior with approximately 5% porosity due to the presence of blood vessels, cells, and canals [31,39]. Trabecular bone is a porous material resembling a network of trabeculae with interconnecting pore spaces filled with red bone marrow. Despite being stiffer, cortical bone is also more fragile than trabecular bone [40]. At the ***micro-structural level*** (500 µm-1 µm), mineralized collagen fibers form planar arrangements called lamellae in both cortical and trabecular bone (3-7 µm) [41]. In cortical bone tissue, lamellae are organized into concentric layers around a central vascular canal (Haversian canal), forming Haversian systems or osteons. Two types of osteons, primary and secondary, are distinguished based on their formation processes [42]. Cement lines, boundaries between secondary osteons and interstitial tissue, play an essential role in the mechanical properties of compact bone, contributing to microstructural heterogeneity and bone toughness. These regions are enriched with non-collagenous proteins and molecules supporting osteoclast-osteoblast coupling during bone remodeling, thus maintaining bone homeostasis and biomechanical integrity [42].

At the ***nano-structural level*** (1 µm-10 nm), the major components of bone tissue include collagen, mineral crystals, non-collagen organic proteins, and water (Figure 3A-B). Osteoblasts deposit collagen molecules, which self-assemble into fibrils. Tiny mineral hydroxyapatite crystals assemble between collagen fibers, resulting in mineralized fibrils as bone tissue matures. These mineral crystals have a flat and elongated shape, arranged parallel to each other in a staggered organization within the bone composite [41].
Nano-scale compositional and mechanical analysis is crucial to help understand the ultrastructural and chemical characteristics of bone in different pathologies, the deformation mechanism of diseased bones, and the achievement of bone repair approaches.

2.2 Bone nano-scale mechanobiological mechanisms

Despite the huge interest in the nano-scale origin of bone damage, there has been a notable dearth of attention directed towards nano-scale mechanobiological mechanisms, primarily due to methodological challenges in visualizing and quantifying them. Consequently, there remains an insufficient understanding of age- or disease-related patterns in osteocyte lacunar pores, with existing data predominantly two-dimensional [43–50]. Despite available data indicating a reduction in lacunar pores in aged human bone, osteocyte canaliculi, which are less than 1 micron in diameter, present a formidable challenge for visualization and quantification, resulting in extremely limited data on their number and volume in human bone [51–53]. Furthermore, the presence of other nano-level porosities in the human bone matrix remains an enigma, as methodological obstacles have impeded thorough assessments, except for permeability studies using tracers of different sizes [54]. The smallest nano-pores are believed to exist between collagen and mineral crystallites, with some potentially filled by bound water molecules [55]. Notably, existing studies have predominantly concentrated on larger pore types, leading to a persistent gap in understanding the hierarchical distribution of bone pores in quantitative studies. Consequently, the relative contribution of smaller pores to overall bone porosity and whether fragile bones exhibit higher porosity at smaller length scales (nano- to micron) remain ambiguous and warrant further investigation (Figure 3C).
Specifically, at the nano-scale, type I collagen molecules self-assemble in fibrils, which are mineralized through the formation of hydroxyapatite within the gaps inside the fibrils; but collagen fibrils also form strong bonds with adjacent fibrils. Crosslinks can be differentiated into enzymatic or covalent crosslinks and non-enzymatic crosslinks or Advanced Glycation End products (AGEs). Both these crosslinks are correlated to bone disorders. The formation of covalent chemical bonds between collagen molecules is vital for the stability of collagen fibres, while AGEs are linked to a decrease in the mechanical strength of collagen fibres [56]. Crystals are characterized by a specific orientation approximately parallel to the long axis of collagen fibres [57] . The interaction of collagen fibres and mineral crystal influence bone quality: orientation of minerals with collagen fibres follows the direction of the primary load, in the trabecular bone the long axis of the mineral and collagen aligns with each lamella, while in the cortical bone different fibril orientation patterns exist. In primary osteons fibrils are oriented in the direction of the osteons, but in secondary osteons there is evidence of varying fibril orientation in different lamellae and osteons [58]. Uniformly oriented bundles of mineralized collagen fibres form the lamella. Most of the lamellae present a twisted plywood pattern, but a limited number of lamellae near the Haversian canal present a fibril rotation like the oscillating plywood pattern [59]. Thin layers of disordered material separate the bundles of collagen fibres, and these thin layers are often the place where the osteocyte processes take place [41] .
Water is another essential component of bone; some water molecules are bound to the surface of the crystals, while others are between the collagen molecules when the mineral is absent or only in part present [41] .
According to different studies [57,60–62], non-collagenous organic proteins (Figure 3B) may play a major role in cell differentiation, cell attachment, and regulation of minerals deposition. Some of these proteins may have different roles in the bone, therefore it is not sufficient to define a single function. Also, some of these proteins may act together causing a synergistic effect on the mechanical properties of bone.

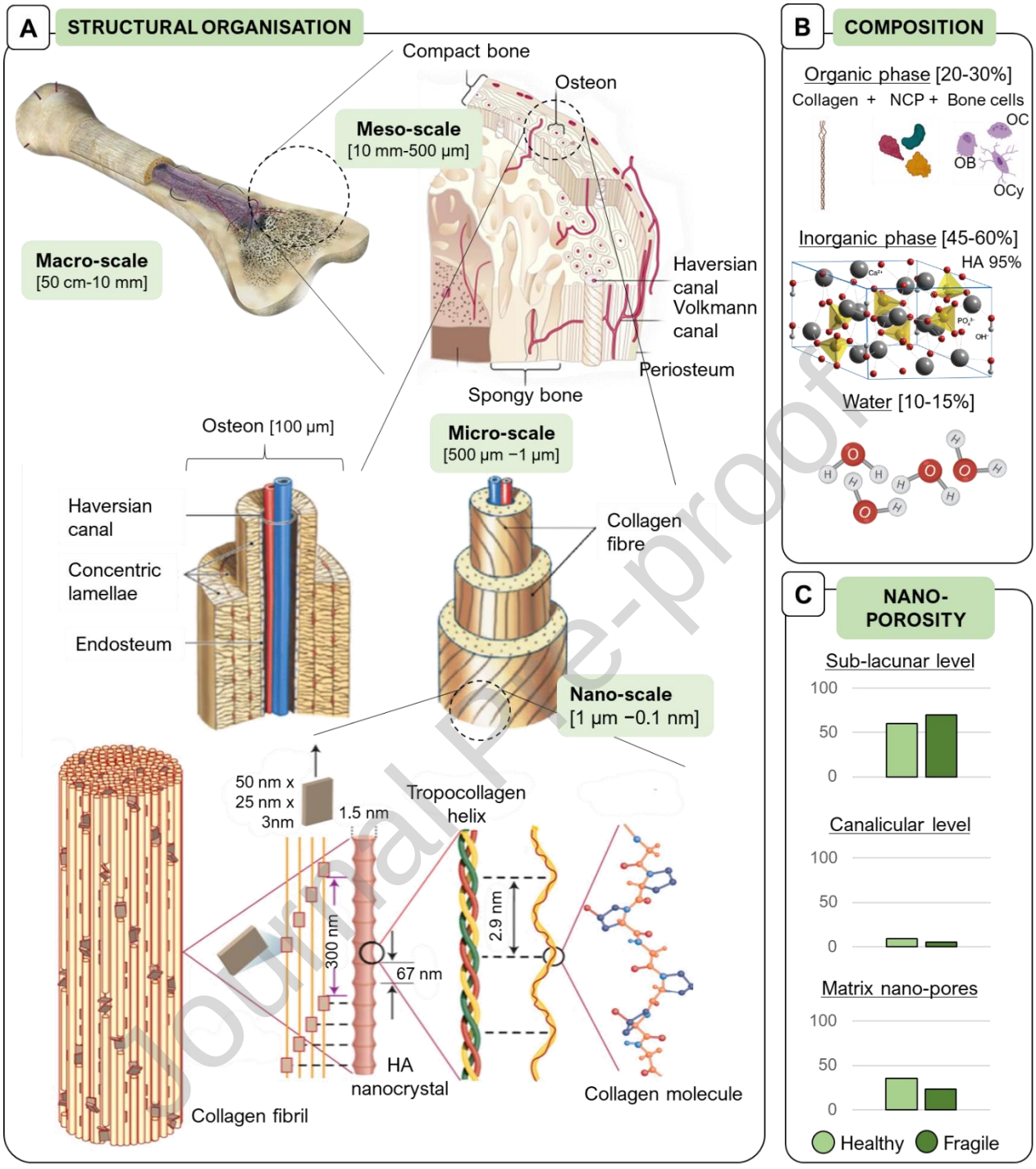

Figure 3| Multi-scale bone organization, composition and porosity with a focus on nano-scale. **A**. Hierarchical organization of bone. At the nano-scale the main components are highlighted and the related dimensions are reported. **B**. Percentages of bone components at the sub-micro and nano-scale. **C**. Nano-porosity level in healthy and fragile subjects from the sub-lacunar scale, to the canalicular and matrix level.

3. Bone nano-scale pathological alterations

In physiological conditions, the intricate hierarchical structure of bone confers resistance to external forces. Nonetheless, bones are susceptible to the effects of aging, environmental factors, and various genetic or metabolic bone diseases, with manifestations becoming apparent at the morphological and structural nano-scale levels (Figure 4). The **aging process and conditions such as osteoporosis** distinctly degrade nano-mechanical properties and the structural integrity of bone tissue. **Aging** contributes to a heightened cross-

link density of collagen molecules and an increased mineral content [63], thereby diminishing energy dissipation before failure and resulting in fractures in elderly individuals [64,65] (Figure 4C). As age and osteoporosis progress, a substantial loss in bone quantity occurs due to a remodeling imbalance favoring resorption over deposition[38,66]. Initial bone loss in osteoporosis primarily affects trabecular bone, transitioning to intracortical loss with advancing age, and bone remodeling predominantly occurs on bone surfaces[67].

At the nano-scale, the integrity of mineral crystals and collagen fibrils is pivotal for overall bone tissue health, and disorders related to these components can precipitate poor bone quality, elevating the risk of catastrophic fractures and other diseases. Various bone diseases, including **osteoporosis** (Figure 4A), genetic disease (as **osteogenesis** imperfecta) (Figure 4B), and **chronic disorders (as Paget's disease)** (Figure 4D), significantly impact the mechanical properties of bone, increasing the likelihood of fractures[68].

Osteoporosis, the most prevalent metabolic bone disease, is characterized by bone material exhibiting a lower elastic modulus and higher tension at failure compared to healthy bone material. Focusing on the nano-scale, no discernible ultrastructural differences are identified between healthy and osteoporotic bone in both the mineral and collagen phases[69]. Notably, the deflection mechanism of microfractures is impaired in osteoporosis compared to healthy bone. In healthy bone, collagen fibrils undergo local disarray around the crack tip, forming significant fibril bridges; however, osteoporotic bone lacks such fibril bridges [70]. Osteoporotic individuals exhibit altered and disorganized collagen, along with a reduction in collagen fiber diameter, contributing to the increased fragility of osteoporotic bone tissue [30,41,71]. Moreover, compositional changes in bones due to osteoporosis involve an increase in mineralization and collagen cross-links, heightening bone fragility and detrimentally affecting mechanical properties, specifically increasing the stiffness while reducing the toughness [72]. Another study indicates that, although overall mineralization in osteoporotic vertebrae is enhanced, the heterogeneity of calcium content distribution compromises bone toughness [73]. This means that while there is an increase in the number of mineral deposits, these are unevenly distributed, leading to regions of high and low mineral density within the bone. Such heterogeneity in mineral content results in variations in bone stiffness and elasticity, making the bone more susceptible to fractures since some areas may be overly brittle while others lack sufficient mineral support to withstand mechanical stress. In osteoporotic patients, the decreased amount of non-collagenous proteins, such as osteopontin and osteocalcin, also contributes to lower fracture toughness. Osteopontin is involved in bone remodeling and the adhesion of osteoclasts to the mineral matrix, playing a crucial role in bone strength and resilience. Osteocalcin, on the other hand, is essential for bone mineralization and calcium ion homeostasis. Lower levels of these proteins impair the bone's structural integrity, reducing its ability to absorb energy and resist fractures. The deficiency in these proteins can lead to a compromised bone matrix, further weakening the bone and increasing the risk of fractures in osteoporotic individuals [74,75].

Osteogenesis imperfecta (OI), or brittle bone disease, is a genetic disorder causing increased bone fragility, low bone mass, and other connective tissue manifestations[76]. In some cases, the disease is linked to mutations in genes encoding collagen type I, while in others, no identifiable mutations are present[77]. Bone tissue in OI patients is extremely brittle due to increased mineralization density and numbers of non-enzymatic cross-links[78]. Mechanical properties studies reveal mechanical weakness in the stiffness and strength of OI tissue[79]. Mutations in OI lead to the development of nano-cracks, inducing changes in fibril stress distribution and locally large shear stresses, predisposing the material to failure[80,81]. Osteogenesis imperfecta (OI) and osteoporosis both lead to significant changes in bone mineral morphology, but they do so through different mechanisms and with distinct characteristics at the nano-scale. In OI, one of the notable changes is the reduction in collagen fibril diameter, together with more wavy, irregular appearance compared to healthy bone [82–84]. This alteration impacts the bone's overall mechanical properties and contributes to its fragility. Additionally, researchers [84–86] have shown that there is an increase in crystal disorientation in OI bone. Bone mineral crystals, specifically hydroxyapatite crystals, are physiologically aligned in a consistent manner that supports bone strength. In OI, the angular spread of these crystals is doubled compared to healthy bone, indicating a greater degree of disorientation. This misalignment can further compromise the structural integrity of the bone and contribute to the increased fracture risk associated with OI.

Regarding bone minerals, lower crystallinity and a reduction in carbonate and phosphate contents have been observed[85,87,88]. This results in a defective mineralization process that, coupled with mineral crystal disorientation, affects the load transfer mechanism between the mineral and collagen phases of bone, leading to poor mechanical performance[89]. However, as most values analyzed in these studies were averaged throughout the entire examined sample, localized alterations necessitate further examination.

The second major affliction following osteoporosis, predominantly impacting individuals aged 50 and above, is **Paget's disease** of the bone. Typically asymptomatic, Paget's disease may present with bone pain and complications such as osteoarthritis, fractures, bone defects, and deafness [90]. This pathology is characterized by excessive bone turnover leading to the formation of structurally abnormal bone [91]. At the nano-scale, Paget's disease exhibits a lower mineral content and greater heterogeneity in mineralized bone packets compared to healthy bone. Nano-scale studies reveal a less organized orientation of collagen fibers, presenting random fibril orientation with increased cross-links, resulting in woven bone formation, that is typically less strong and more pliable than the lamellar bone found in healthy bone [92]. Concerning the mineral phase, Paget's disease typically induces a reduction in the mean mineralization of bone [93]. Utilizing a combination of small-angle and wide-angle X-ray scattering techniques, the spatially heterogeneous distribution of mineral orientation angles and disorganized mineral arrangement has been elucidated [94]. A delicate balance between diminished bone quality and heightened intrinsic hardening through ductile plastic deformation, attributed to a higher collagen/mineral ratio, has been hypothesized [68,92].

As an additional nano-scale fragility factor, in 2020, the world experienced the global impact of the coronavirus disease (**COVID-19),** officially declared a pandemic. COVID-19 infection manifests with pulmonary and extrapulmonary symptoms, and recent studies have delved into the direct and indirect effects of the virus on bone multi-scale architecture. Indeed, a slowdown in the bone formation processes is hypothesized as a direct consequence of the infection (Figure 4E), which, together with the increased sedentary lifestyle of the bedridden hospitalized patients, lead to a reduction in bone mass and strength [95,96]. Patients with COVID-19 in intensive care demonstrated lower bone mineral density (BMD) compared to those without intensive care [97]. The infection induces bone loss through heightened production of inflammatory cytokines, shifting the balance toward osteoclasts, leading to increased bone resorption [98]. Several studies have explored the relationship between COVID-19 and traumatic bone fractures. A recent multi-center, retrospective, epidemiological study conducted at three healthcare centers in Turkey reported an increase in adult proximal femoral and hand fractures, as well as pediatric distal forearm fractures during the pandemic period [99]. Ongoing research is evaluating the consequences of COVID-19 on bone biomechanics at smaller scales, demonstrating altered sub-micro scale pore morpho-densitometric characteristics and reduced lacuna-canalicular interconnectivity.

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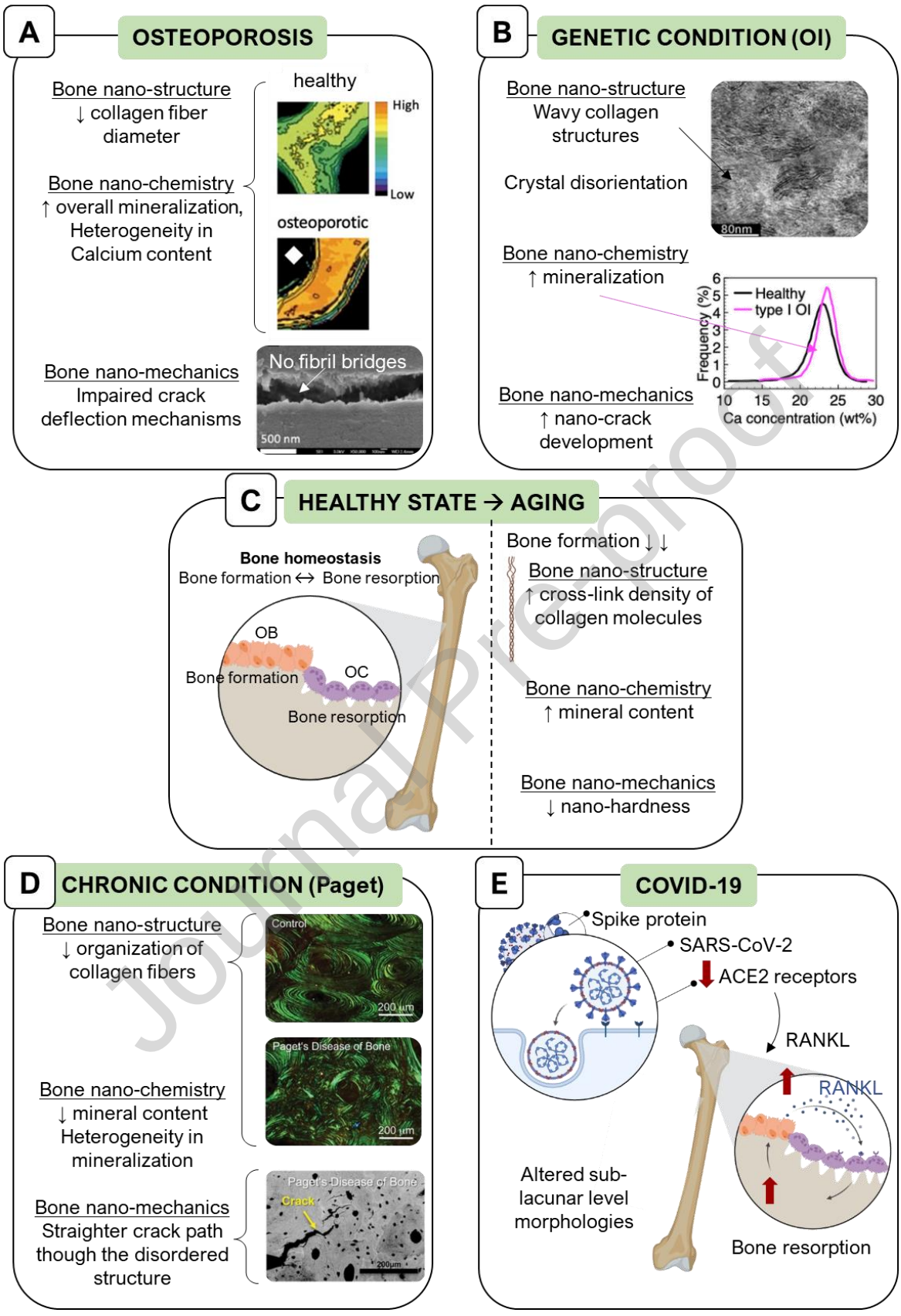

Figure 4| Bone nano-scale fragility factors, including aging (as a physiological process), bone-related pathology (i.e. osteoporosis), genetic condition (i.e. osteogenesis imperfecta), chronic condition (i.e. Paget disease), and Covid-19. **A**. Osteoporosis modifications on bone nano-structure, nano-chemistry and nano-mechanics. **B**. Osteogenesis Imperfecta (OI) modifications on bone nano-structure, nano-chemistry and nano-mechanics. **C**. Aging modifications on bone nano-structure, nano-chemistry and nano-mechanics. **D**.

Paget disease modifications on bone nano-structure, nano-chemistry and nano-mechanics **E**. Effect of Covid-19 on bone remodeling processes at the micro-scale. The downregulation of the Angiotensin-Converting Enzyme 2 (ACE2) is schematized. Images is adapted with permission from [100].

4. Advances in nanoscopic imaging techniques to predict pathological alterations

In recent years, the field of bone fragility prediction has witnessed transformative advancements due to cutting-edge nano-scale imaging techniques (Table 1), providing unprecedented precision and depth. High-resolution transmission electron microscopy (HR-TEM) and scanning electron microscopy (SEM) have proven indispensable, allowing researchers to identify bone ultrastructure with nanometer-scale resolution. HR-TEM, with a resolution approaching 0.1 nm, has enabled the visualization of mineralized collagen fibers, their spatial organization and orientation within the bone matrix. SEM, with resolutions in the range of 1-10 nm, complements this by offering detailed surface imaging. When used with specialized sample preparation (i.e. cryogenic conditions to immobilize thin specimens in cryo-TEM) or other techniques (Focused Ion Beam (FIB) in FIB-SEM), electron microscopy enables in situ bioimaging of bone without compromising much of the spatial or temporal resolutions.

Synchrotron X-ray microtomography, a core tool in three-dimensional imaging, provides spatial resolutions down to 100 nm, offering reconstructions of bone architecture. This technique has become crucial in understanding the nano-scale trabecular architecture, cortical bone density, and the distribution of mineralized components. Atomic force microscopy (AFM), with its high sensitivity and resolution down to sub-nanometer scales, facilitates precise probing of surface morphology and mechanical properties (specified in paragraph 5) at the nano-scale. AFM's nano-indentation capabilities provide quantitative data on bone hardness and elasticity.

Quantitatively, these techniques have boosted the understanding of nano-scale alterations in bone morphology, revealing aspects as collagen fibril diameters (approximately 100 nm) and mineral crystal dimensions (in the order of nanometers). The integration of these state-of-the-art imaging techniques and their complementarity (see Figure 5) has not only contributed to fundamental research in bone biology but has also provided quantitative insights into nano-scale variations in bone composition and mechanical behavior. As these imaging technologies continue to evolve, the spatial resolution and quantitative capabilities are expected to further refine, promising continued advancements in the understanding and application of nano-scale bone analysis for diagnostic and therapeutic purposes.

Table 1. Nano-scale imaging techniques, related information, pros and cons and invasiveness.

Nano-scale Imaging techniques	Application	Advantages	Disadvantages	Invasiveness	References
Laser Scanning confocal Microscopy (LSCM)	Assessment of micro-damage to bone	Bone micro-damage assessment	Axial resolution in depth impaired by spherical aberration High costs	Yes	[101,102]

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Scanning Electron Microscopy (SEM)	Sub-micro-scale damage visualisation Visualisation of osteons and the cement line delimiting osteonal and interstitial bones	Relevant information on sub-micro-scale damage	Destructive technique - the surfaces of bone samples must be conductive	Yes	
Atomic Force Microscopy **(**AFM)	Fracture surfaces and sacrificial bonds visualisation	Adaptable imaging technique for the visualisation of fracture surfaces High accuracy Physiological conditions Non-destructive technique	Small dimensions of the single scan image size (150 × 150 µm, compared with mm for SEM) Slow scan time	Yes	[103,104]
Micro-MRI and nano-MRI	Structural parameters of bone tissue structure and fracture resistance	Non-destructive technique Good special resolution Good contrast resolution costs	Long acquisition times High costs	No	[105]
Raman spectroscopy	Mineral composition, collagen structure, and molecular components of bone for bone quality and integrity assessment	High sensitivity Non-destructiveness High specificity	Time-consuming sample preparation Limited penetration depth High equipment costs	No	[106]

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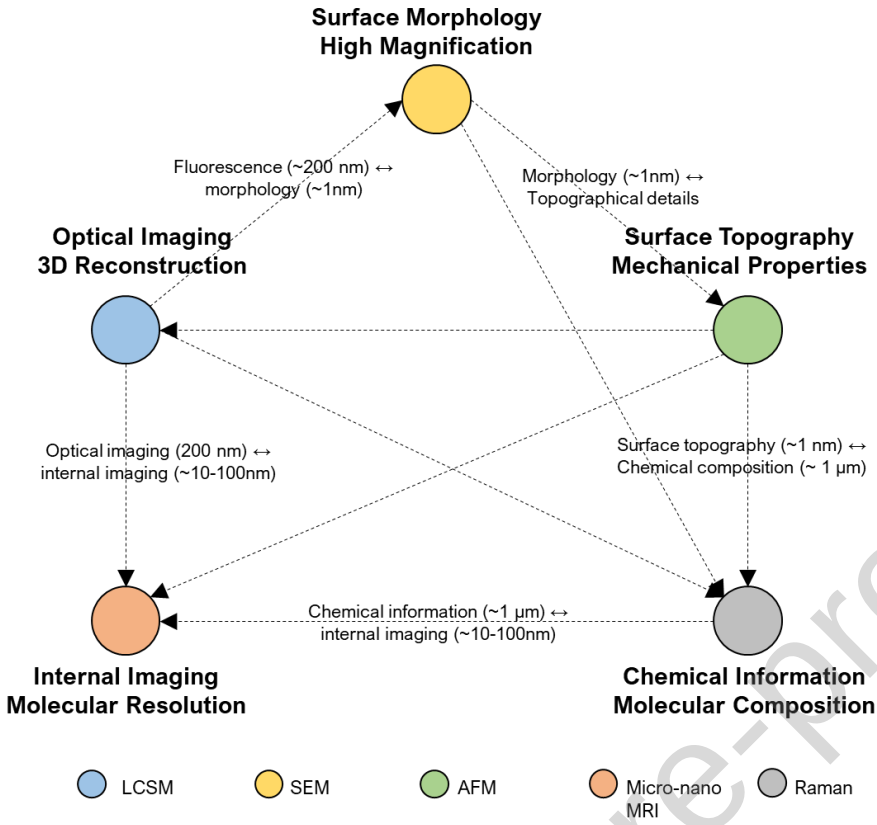

Figure 5| Different nano-scale imaging techniques provide unique and complementary information.

5. Nano-mechanical characterization to predict pathological alterations

5.1 Bone multi-scale mechanical response and interactions

Bones sustain diverse mechanical loads during both routine activities and traumatic incidents such as falls, adapting its architecture as a response to the loading scenario. These intricate mechanobiological phenomena occur at the multi-scale; moreover, changes in nano-scale architecture, triggered in response to mechanical loading, resonate across larger scales. Understanding the nano-mechanical properties of bones is imperative for clinical studies and for unraveling the nano-mechanobiological mechanisms underpinning bone diseases.

In the normal functioning of the skeletal system, bone tissues experience a combination of normal and shear stresses[107]. ***Bone toughness***, denoting its resistance to fracture, is intricately tied to the adjustments and interactions of its material and structural properties across different length scales within its complex and hierarchical structure [30,31].

Accurate assessment of ***bone tissue multi-scale stiffness*** is crucial for comprehending the impact of factors such as disease and age on bone quality[108]. Stiffness, representing the resistance of a structure or material to elastic deformation, is quantified by the elastic modulus, which varies at each hierarchical level of bone structure.

Bone tissue exhibits rapid responses to structural changes and metabolic needs through remodeling, a phenomenon studied formally since 1892 under Wolff's law. This law posits that bones adapt to mechanical loading [109], resulting in the strengthening of the internal trabecular architecture and the cortical layer with increased loading, and weakening under reduced stress [110].

As a ***viscoelastic material***, bone mechanical behavior varies with the rate and duration of applied loading force[111]. This rate-dependent failure behavior, or viscoelasticity, is a pertinent factor in bone damage and fracture. While trabecular and cortical bone share similar creep characteristics, cortical bone is more fragile at high strain rates [112–115]. It is possible to show with micropillar compression at varying strain rates that the strain rate sensitivity at the microscale is equal to the one at the macroscale and it is therefore an inherent property of the extracellular matrix[116]. Cement lines may contribute to bone viscosity [117]. Hydration

significantly influences the viscoelastic properties of bone, with the relaxation time constant positively correlated to water content in torsion. Hydrated bone exhibits increased viscoelastic damping compared to dry bone across a broad range of frequencies [118].

Being an ***anisotropic material***, the elastic modulus of bone varies with the direction of the applied load. Cortical bone is nearly transversely isotropic, while trabecular bone is often orthotropic [119]. The mechanical response to loading differs significantly between cortical and trabecular bone. Factors such as osteons, porosity, mineralization, density, architecture, and collagen fiber organization affect the mechanical properties of cortical bone, with lamellar interfaces promoting toughness [112,113]. Studies have indicated that collagen fiber orientation influences the mechanical properties of individual osteons, enhancing the bone ability to support different types of stress [120]. Lacunae act as both stress concentrators and crack deviators, affecting bone strength, and changes in their distribution and shape with aging significantly impact bone resistance to fracture [121]. Sub-microdamage typically initiates at cement lines or canals where stress amplification is greater than at the lacunae[68]. An increase in mineral content is associated with decreased fracture toughness. Given the limited change in porosity and mineralization in cortical bone, collagen fiber orientation may be the predominant factor influencing tensile strength [114,122]. As pertains cancellous bone, the porosity influences the variability of the elastic modulus, thereby impacting its stiffness and strength. In detail, the modulus and compressive strength of individual bone lamellae is measured using micropillar compression combined with Raman spectroscopy [123]. Fibril orientation also clearly influences tensile strength [124].

Anisotropy is determined by trabecular orientation in trabecular bone, whereas cortical bone is primarily governed by lamellar and osteonal orientation. The orientation, numbers, and sizes of trabeculae control anisotropy, influencing the elastic modulus and failure stress [58,125,126],. Microdamage tends to increase with age, depending on architectural parameters such as orientation and local thickness of trabeculae [127].

At the **nano-scale**, mechanical properties are influenced by the specific arrangements and mechanical interactions between mineral crystals and collagen fibrils [114]. The primary deformation mechanisms in collagen fibrils involve intermolecular scrolling and breaking of cross-links between collagen molecules. Notably, collagen exhibits greater resistance to deformation compared to mineral crystals. The stress distribution between collagen and minerals enable an energy dissipation mechanism, contributing to the bone resistance to fractures [33,114]. Further elucidation of these nano-scale mechanisms is presented in the subsequent paragraph (Section 3.3). The examination of bone tissue at the nano-scale holds significant importance, particularly for advancing and facilitating early diagnoses of fragility fractures. However, investigating tissue structure and its mechanical properties at the nano-scale remains a challenging endeavor.

5.2 Nano-scale mechanical behaviour of physio-pathological bone: techniques and challenges

Understanding nano-scale mechanisms provides insights into damage mechanisms at higher hierarchical levels of bone structure, including the macro-scale. At the nano-scale, bone fractures are influenced by compression-resistant hydroxyapatite minerals and tension-resistant collagen fibers. Examining parameters such as collagen fiber diameter, orientation, mineral crystal size, and orientation is of paramount relevance, as they contribute to the fragility of bone tissue [12].

In healthy bone tissue, at the lamellar level, collagen fibrils exhibit a parallel and highly oriented arrangement to enhance mechanical properties. Overall, lamellar orientations may vary significantly to address the continuous evolving loading scenario. Primary deformation mechanisms of single collagen molecules involve molecular stretching, unwinding, and breaking of hydrogen bonds, along with sliding movements between molecules. These sliding movements facilitate substantial plastic strains without catastrophic brittle fractures [64,128].

The orientation of mineral crystals within collagen fibrils plays a crucial role in determining how bone tissue withstands loads in different directions, with studies demonstrating their parallel arrangement within the bone matrix [69,129,130]. Mineralized collagen fibrils are integral for bone strength, providing resistance against plastic deformation, increased elastic modulus, and fracture resistance. Under significant loads, adhesion forces between mineral crystals and collagen fibers allow sliding movements, strengthening bone without inducing fractures in collagen molecules [64,131].

Beyond collagen fibers and minerals, bone encompasses a protein-based "glue" involving sacrificial bonds and a hidden length system. Covalent and non-covalent bonds bind mineral collagen fibrils within a "glue layer," potentially playing a significant mechanical role [132]. "Sacrificial bonds" are reformable bonds in the organic component, enhancing fracture toughness through molecular-scale energy dissipation [57,133]. Under stress conditions, some sacrificial bonds break, allowing considerable length increase with high energy absorption. In bone, multivalent positive ions like calcium enhance the sacrificial bond-hidden length system. Consequently, non-collagenous bone matrix proteins with negative charges at physiological pH can be bound together into sacrificial bonds[132].
The Table 2 summarizes the main techniques employed for investigating the mechanical properties of bone at the nano-scale, including their real-word applications for elucidating early pathological signs.

Table 2. The main techniques employed to analyse bone mechanical properties at the nano-scale.

Characterization techniques	**Main applications & Information on early disease state**	**Advantages**	**Disadvantages**	**Real-World Applications**	**Ref.**
Nanoindentation	Measures hardness, elastic modulus, creep parameters, loss and storage modulus, fracture toughness, and residual stress. Provides high-resolution data on the mechanical properties of bone at the nano-scale, useful for detecting early changes in mineralization and matrix composition associated with diseases like osteoporosis, OI.	***High spatial resolution***: single to multiple lamellae ***Comprehensive data***: simultaneous measurement of multiple properties ***Non-destructive***: preserves the sample for further analysis.	**Limited yield property evaluation**: less effective for yield properties and plastic deformation. **Complex data interpretation**: requires sophisticated data analysis. **Small scale**: may not capture bulk material properties.	***Osteoporosis studies***: research on how bone mineral density and matrix properties change with osteoporosis. ***Bone Quality in OI***: investigations into how collagen matrix abnormalities affect bone mechanics.	[63,134–136]

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Micropillar compression	Insights into mechanical properties such as elastic modulus, yield stress, plastic deformation, and damage accumulation by compressing small, pillar-like structures. Useful for assessing localized mechanical behavior and understanding the effects of early disease states on bone's structural integrity.	***Direct measurements***: straightforward assessment of mechanical properties. ***Detailed failure analysis***: information on damage accumulation and failure mechanisms. ***Adaptable***: used for various materials and structures.	***Specimen preparation***: meticulous preparation, time-consuming. ***Potential for artifacts***: preparation might introduce artifacts or errors.	***Osteoporosis studies***: analysis of localized changes in bone nano-structure. ***Bone nano-structure in Paget's disease***: evaluations of changes in mechanical properties of bone pillars in Paget's disease.	[89,92,137,138]
Microcantilever bending	Evaluation of toughness and bending resistance of bone tissue by measuring the deflection of a cantilever beam. Useful for assessing single trabecular structures and changes in mechanical properties due to early disease states.	***High-Resolution measurements***: precise deflection and loading measurements	**Sample preparation and measurements**: complete isolation of the sample, deflection measurements at high resolution and extremely precise loading	***Osteoporosis studies***: measuring changes in trabecular bone stiffness and toughness. ***Bone Quality Assessment in OI:*** evaluating trabecular bone properties in OI models.	[139–141]

Nanoindentation stands out as a prominent experimental technique for assessing bone mechanical properties at the nano-scale. Primarily employed for measuring hardness and elastic modulus, nanoindentation offers versatility by extracting various mechanical parameters such as creep parameters, loss and storage modulus, and fracture toughness [142]. This method evaluates the surface of the material under test with high lateral resolution, facilitating specific surface mapping and the identification of local variations in mechanical properties within thin, small, and heterogeneous bone samples. The nanoindentation process involves applying a known force with a hard tip onto a flat surface of the analyzed sample while measuring tip displacement (h) and reaction force (F) [124]. The resulting force-displacement curve enables the extraction of mechanical properties like elastic modulus and hardness, following the method proposed by Oliver and Pharr [143].

Elastic-plastic materials exhibit permanent deformation during the loading phase, complicating the interpretation of the load-displacement curve. However, during the unloading curve's initial segment, behavior is considered purely elastic, allowing for easier interpretation. The unloading stiffness (S) is derived from the slope of the force-displacement curve, computed at the maximum nanoindentation depth:

$$S = (dF/dh)_{h=hmax}$$

The reduced modulus E_r is then calculated using the general relationship [143]:

$$E_r = \frac{\sqrt{\pi}}{2} S \frac{1}{\sqrt{A_c}}$$

Where A_c is the projected contact area, dependent on the contact depth and the geometry of the indenter tip. The elastic modulus E can be determined from the reduced modulus E_r using the relationship:

$$\frac{1}{E_r} = \frac{1 - \upsilon^2}{E} - \frac{1 - \upsilon_i^2}{E_i}$$

Considering known isotropic constants E_i and υ_i of the indenter tip and υ as the Poisson's ratio of the tested material. The indentation hardness H_{ind} can also be calculated using the Oliver-Pharr method, defined as the maximum force divided by the contact area at maximum depth:

$$H_{ind} = \frac{F_{max}}{A_{c,max}}$$

The Oliver-Pharr method assumes a linear isotropic solid with time-independent post-yield behavior and a known Poisson's ratio. While improved techniques for nanoindentation have been introduced, the complex stress state below the tip remains a major limitation, especially for anisotropic and heterogeneous materials as bone with unknown dissipative mechanisms [144].

Another high-performance technique for nano-mechanical characterization, particularly of biological samples, is nanoindentation with atomic force microscopy. This characterization has implications for early disease diagnosis, including cancer and osteoarthritis [145].

Recent studies have emphasized the importance of determining the elastic modulus of collagen fibers at the nano-scale, as alterations in collagen fibers correlate with various diseases. Factors contributing to the variability in elastic modulus values include the dehydration state of the fibril, errors in data processing, and uncertainties associated with AFM probe calibration processes [146–148]. Acknowledging the challenge of explaining the mechanical heterogeneity of samples with a single elastic modulus value, the average elastic modulus emerges as a relevant physical quantity for describing collagen fibrils mechanical properties [149].

A complementary experimental technique, ***micropillar compression***, introduced recently by Uchic et al. in the early 2000s [150], offers valuable insights into the micromechanical properties of bone tissue. Distinct from nanoindentation, micropillar compression employs a flat punch indenter to compress a small volume of material with a defined geometry, typically a rectangular or cylindrical pillar, instead of compressing a flat surface with a sharp tip. This approach generates a relatively uniaxial and uniform stress-strain field in the tested volume, maintaining a constant area of contact throughout the experiment and allowing for a simpler assessment of elastic and post-yield properties at a micrometer scale compared to nanoindentation.

The stress (σ) and strain (ε) in the pillar can be calculated as follows:

$$\sigma = \frac{F}{A}$$

$$\varepsilon = \frac{\Delta l}{l_0}$$

Here, F is the measured force, A is the cross-sectional area of the pillar, Δl is the indenter displacement, and l_0 is the initial height of the pillar. Accounting for the pillar sinking effect due to substrate deformation, Δl can be determined from the tip displacement $l_{measured}$ using the modified Sneddon correction proposed by Zhang et al [151].

During data analysis, it is crucial to consider indenter frame compliance and pillar sink-in effects. For a cylindrical pillar manufactured on a bulk sample, the incremental displacement Δl is given by:

$$\Delta l = l_{measured}\left(\frac{-2l_0 a_c}{A(\upsilon^2 - 1) - 2l_0 a_c}\right)$$

Here, υ is the Poisson's ratio of the sample material, a_c indicates the area of contact between the sinking pillar and the substrate, and can be expressed as:

$$a_c = \mu(r + r_c)$$

where r is the radius of the pillar, r_c is the radius of curvature at the base of the pillar, and μ is a constant term (μ=1) using Sneddon's method [152], (μ = 1.42) using Zhang's method [151]).

While micropillar compression tests offer more straightforward data analysis than nanoindentation, specimen preparation is more challenging, requiring appropriate instrumentation and being time-consuming. For bone specimens, focus ion beam (FIB) milling is the only suitable fabrication technique for the generation of small pillars; while for larger pillars, FS laser ablation is also possible [145] Specifically, this technique, combined with nanoindentation and Raman spectroscopy, allows to screen micromechanical properties in osteoporotic patients [153].

Concerning bone-focused applications, the micropillar compression technique is extensively employed to investigate the micromechanical properties of tissues, including elastic modulus, yield stress, plastic deformation, damage accumulation, and failure mechanisms. Notably, there is a growing interest in correlating bone microstructure to macro-mechanical properties, aiming to understand potential differences between these two scales. In a seminal in situ micropillar compression study [154], it was demonstrated that, apart from consistent elastic properties, post-yield properties, and failure mechanisms of bone under compression significantly differ between macro and micro-scales. Another study [137], utilizing micropillar compression tests, highlighted the influence of hydration on microscale yield properties, showing a 60% reduction compared to dry conditions [116,123].

In the realm of assessing bone nano-mechanical properties through nanoindentation and micropillar compression techniques, the challenges associated with ***sample preparation and fabrication processes*** remain highly intricate and demand meticulous attention, given their potential adverse effects on bone mechanical properties. A pivotal concern is sample handling, as manipulating miniaturized samples poses significant complexity and the risk of premature damage [155,156]. Such concerns, if unaddressed, can impede the elucidation of early signs of fragility [157]. To mitigate these challenges, various approaches, including chip fabrication and co-fabrication of both the sample and testing setup, have been employed [158–160]. However, these techniques are subject to intricate setup management and material-specific manufacturing methods; besides, heterogeneous deformations may introduce measurement errors when applying microscopic samples to a testing setup using adhesive methods [161]. Additionally, clamping procedures can exert high forces at the edges of the bone sample, potentially inducing failure in the initial stages of mechanical testing. Misfits between the sample and gripping surfaces might lead to unwanted bending [162]. It is imperative to ensure optimal alignment between the sample and tensile setup to prevent significant errors and critical issues in micromechanical testing [151,163,164]. Furthermore, the design of the sample should incorporate smooth transition zones to avoid premature failure caused by stress concentrations [165]. The integration of nanoindentation setups with FIB fabrication techniques holds

promise for developing a method capable of characterizing the microtensile properties of bone at the lamellar scale. A notable study by [138] conducted micro-tensile testing coupled with post-test Scanning Transmission Electron Microscopy (STEM) observation to scrutinize bone nano-deformation and failure mechanisms. The study aimed to establish a failure model capable of predicting strength and failure mode based on mineralized collagen fiber orientation. The findings revealed a brittle micromechanical response, indicating significantly higher ultimate tensile strength compared to the macroscale (a factor of 2.3). Moreover, the study uncovered a substantially greater strength anisotropy in tension compared to compression [124], even if there is a significant effect of sample size and hydration on the micro-tensile behaviour [166].

As an additional method to elucidate bone nano-mechanical characteristics and fragility signs, ***microcantilever bending*** serves as a valuable tool, allowing for evaluating the toughness. It permits precise measurements at specific locations to unveil the impact on thin films and other factors associated with the local fracture toughness of the material [139]. In a study by[140], notched microcantilever bending experiments are conducted in conjunction with finite element (FE) simulations, leading to the determination of critical fracture loads and characteristic toughness values for the examined grain boundaries.

An innovative experimental method to explore the elastic properties of a single trabecula based on cantilever bending is demonstrated by [167]. The micro-cantilever bending test offers the advantage of easy sample fixation without the necessity for complete isolation from the trabecular bone [167]. However, despite the potential, no significant correlation is observed between the estimated elastic modulus and trabecular orientation, size, or shape. Three-point bending tests on a single trabecula are also conducted in various studies [168–172]; nevertheless, these tests often require complete isolation of the sample, deflection measurements at high resolution, and exceedingly precise loading, presenting challenges in practical implementation.

In conclusion, nano-scale compositional and mechanical analysis play a crucial role in comprehending the ultrastructural and chemical characteristics of bone in different pathologies, understanding the deformation mechanisms of diseased bones, and advancing bone fragility prediction approaches.
However, the combination of mechanical testing and high-resolution imaging, given the impressive dataset these techniques provide, are not sufficient to provide insights on bone early damage without the aid of nano-scale numerical modelling.

6. Nano-scale numerical modeling to predict pathological alterations

Modeling bone at the sub-microscale and nano-scale involves examining individual mineralized collagen fibrils and their arrangement, leading to the identification of crack formation at its early stages. Herein, it is possible to consider two main categories: finite element (FE)-based computational models (2D and 3D) molecular dynamics-based computational models (Figure 6).

FE-based computational models

The majority of finite-element method (FEM) analyses examining bone deformation and failure at the nano-scale typically employ a **two-dimensional** representation (Figure 6A) of the staggered arrangement of mineral platelets within a collagen matrix, as initially proposed by Jäger & Fratzl [173]. For instance, Siegmund et al. [174,175] utilized a cohesive FEM model incorporating a traction-separation law to explore the impact of interfaces and collagen cross-linking on the stiffness and strength of a mineralized collagen fibril. This study considered enzymatic and non-enzymatic cross-links, revealing that the latter increases stiffness and decreases toughness in bone, while the former has minimal effects on the mechanical properties of a mineralized collagen fibril. Luo et al. [176]also adopted a cohesive FEM model to analyze the influence of mineral–collagen interfacial behavior on microdamage progression in bone, incorporating three interface types: strong, intermediate, and weak. Cohesive finite element modeling studies on regular 2D matrices of mineral and collagen demonstrated that microdamage patterns could be influenced by interfacial properties between mineral and collagen phases [176] . Strong surfaces promoted the formation of linear microcracks,

while weak surfaces led to diffuse damage. Additionally, the cohesive finite element model assessed the contribution of the extra-fibrillar matrix to bone mechanical behavior [177]. Moreover, 2D models of various extra-fibrillar matrices (EFMs) underscored the substantial contribution of EFMs to pre-stress deformation, revealing the formation of nanocrystals within the EFM and progressive load transfer until failure[178].

Hambli and colleagues [179–181] propose a **three-dimensional** model for a mineralized collagen fibril, comprising five Triple helical Collagen (TC) molecules, shifted at intervals of 67 nm to form a cylindrical shape, with the space between TC molecules and non-collagenous proteins (NCPs) filled with a mineral phase. Collagen cross-links are represented as linear springs, and potential sliding at interfaces is disregarded. The study investigates the impact of the number of cross-links, Young's modulus of hydroxyapatite (HA), and the HA volume fraction on failure properties, encompassing damping capacity and fracture stress at crack initiation (Figure 6B). Results indicate that a higher number of cross-links leads to significantly increased damping capacity and fracture stress, up to 20 cross-links, beyond which additional cross-links have negligible effects. Moreover, an increase in mineral stiffness elevates fracture stress with no substantial alteration in damping capacity, while a higher HA volume fraction results in lower fracture stress and damping capacity. This model, akin to their earlier studies, is employed to comprehensively explore the elastic properties of bone [182–185]. Another 3D model, utilizing a fracture mechanics approach, introduces a new set of nodes at crack formation points and indicate that the denaturation of non-collagenous proteins at mineral-matrix interfaces significantly contributes to bone toughness [186].

Molecular dynamics-based computational models

Conducting experimental studies at the fundamental level of bone components, such as collagen molecules and hydroxyapatite (HA) crystals, presents significant challenges [187–190] (Figure 6C). Consequently, atomistic-level simulations have been extensively utilized [191] to investigate deformation mechanisms and failure at the nano-scale, encompassing single collagen molecules, bundles of collagen molecules, cross-linked collagen molecules, HA crystals, and collagen–HA systems [192–201], employing molecular dynamics (MD) simulations. Pioneering comprehensive studies at the nano-scale, Buehler and colleagues explore the mechanical properties of a single triple helical collagen (TC) molecule and bundles of TC molecules under various loading conditions, assessing fracture strength through atomic-scale simulations [191]. Subsequently, their investigations extended to cross-linked collagen fibrils under significant strain deformations, revealing that increased cross-link density leads to higher strength, albeit with a more brittle behavior [202]. Nair et al. [199] introduced a three-dimensional molecular structure model of a mineralized collagen fibril with mineral densities ranging from 0% to 40%, examining its mechanical properties and identifying deformation mechanisms. Their findings highlighted collagen's predominant contribution to the deformation response, with minerals bearing four times higher stresses than collagen.

Dubey and Tomar [203–209] conducted MD simulations on collagen–mineral systems, extensively exploring the strength of these structures. Employing a staggered arrangement of HA crystals embedded in a TC matrix, they investigated parameters such as TC–HA interfacial arrangement, environmental conditions (presence or absence of water and calcium ions), loading direction, shape of HA crystals, and disease effects [203–209].

MD simulations are also employed to study the impact of geometric confinement on the mechanical properties of bone constituents. Varying crystal height while maintaining width at approximately 30.1 nm and out-of-plane thickness at approximately 2.1 nm, it is observed that [210] for samples with a height of 4.15 nm or smaller, stress concentration at the crack tip disappeared, resulting in a more ductile failure mode, approaching the strength of a flawless section. Another study by Libonati et al. [198]investigated the influence of confinement and water presence on the behavior of the HA–collagen interface. Their observations indicated that final failure occurred through TC molecule breakage rather than interface failure, with deformation mechanisms involving the breaking of atomic interactions, uncoiling and unfolding of collagen chains, and sliding of collagen on the HA surface due to the formation and breakage of H-bonds.

In conclusion, numerical modeling at the nano-scale offers a multitude of advantages, providing a powerful lens through which researchers can unravel the intricate behaviors of bone at the molecular and atomic levels. One significant advantage lies in the ability to simulate and analyze phenomena that are challenging, if not impossible, to observe experimentally due to their scale. The mentioned numerical models facilitate

the rapid exploration of a wide range of parameters, understanding of how changes at the nano-scale propagate to larger scales.

However, nano-scale modeling comes with several inherent limitations. Indeed, the biological intricacies of systems like bone constituents, including collagen fibers and hydroxyapatite crystals, necessitate simplifications in simulations, potentially leading to an oversimplified representation of the complex reality. The computational demands for nano-scale simulations, both in terms of processing power and memory, present formidable challenges for achieving large-scale or long-duration simulations. Furthermore, the accuracy of these models heavily relies on the quality of force fields, and experimental validation is often hindered by the scarcity of comprehensive nano-scale data. Size effects, quantum phenomena, and the intricacies of defining appropriate boundary conditions contribute to the complexities involved in accurately representing material behaviors. As researchers increasingly delve into nano-scale modeling, a nuanced awareness of these limitations becomes imperative, emphasizing the importance of exercising caution in the interpretation of results and highlighting the necessity of integrating experimental data to enhance the reliability and applicability of computational findings. Recently, the computational cost burden has been significantly decreased by the implementation of artificial intelligence tools, exploited to predict damage evolution from the automatic recognition of determinants of nano-scale damage. Those methods, however, are still at their infancy and the difficulty to provide timely experimental validation represent an evident obstacle for their translation to larger scales up to the clinics.

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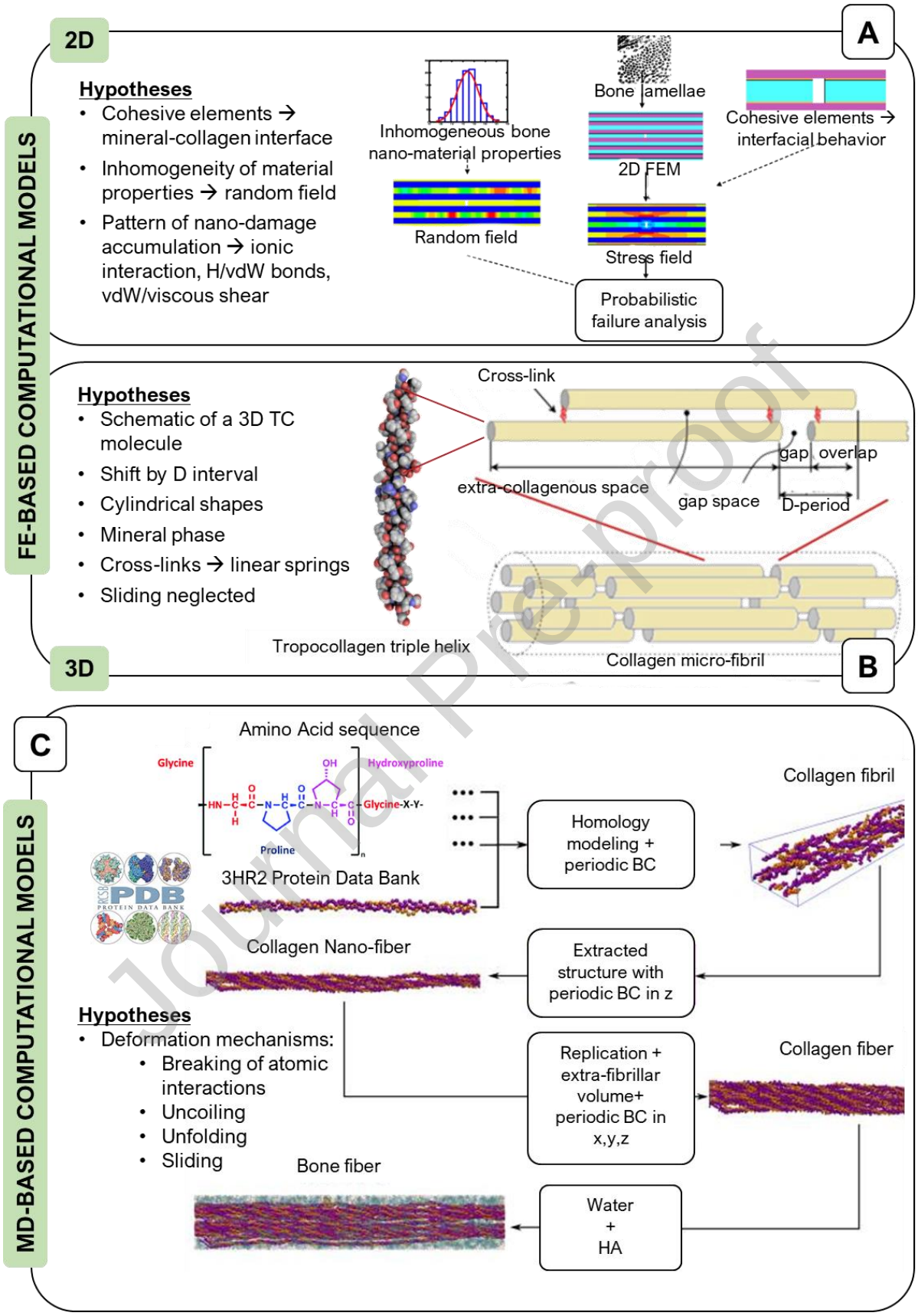

Figure 6| Nano-scale numerical modelling approaches, including FE-based strategies, and MD-based approaches. **A**. 2D finite element-based strategies and underlying hypotheses. **B**. 3D finite element-based strategies and underlying hypotheses. **C**. molecular dynamics-based strategies and underlying hypotheses..

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7. Prospective outlook: from nano-scale to the clinics

The integration of nano-scale imaging, nano-mechanical characterization, and sophisticated computational modeling represents a groundbreaking approach in the study and treatment of bone fragility, unveiling a new era of precision medicine for bone health [70].

Traditional therapeutic methods often rely on high-dose, extended medication regimens that fall short in addressing the nano-scale origins of bone damage due to the dense and complex nature of bone tissue, which hampers effective drug delivery. This challenge is being addressed by ***emerging technologies*** that promise to revolutionize our approach to bone disease management. Advanced imaging techniques, such as synchrotron radiation and cryo-electron tomography, are pushing the boundaries of resolution, allowing researchers to visualize bone structures at unprecedented detail. These technologies provide critical insights into the organization of mineralized collagen fibrils, the distribution of cross-links, and the presence of micro-damages that are pivotal for understanding the mechanisms of bone fragility.

Additionally, the convergence of machine learning and artificial intelligence with imaging data is set to transform how we analyze and interpret complex datasets, enabling more accurate predictions of fracture risk and treatment responses. These AI-driven models can process vast amounts of imaging data, identifying patterns and correlations that may be imperceptible to traditional methods. This advancement facilitates the development of highly personalized treatment plans, tailored to the specific characteristics of an individual's bone structure and pathology.

Nanotechnology is at the forefront of this transformation, offering innovative solutions for drug delivery and therapy. Multifunctional nano-materials are being developed to simultaneously provide imaging, drug delivery, and therapeutic interventions. These materials are engineered to target bone-specific sites with high precision, delivering drugs in a controlled manner while providing real-time imaging feedback on treatment efficacy [211]. Stimuli-responsive nanocarriers, which release their payload in response to changes in the local biological environment or external triggers, represent another significant advancement, enhancing the specificity and effectiveness of treatments.

Furthermore, bioengineered scaffolds incorporating advanced nanomaterials are being designed not only to support bone regeneration but also to deliver therapeutic factors directly to sites of damage [211]. These smart scaffolds can address early signs of nano-scale damage and promote healing by providing a localized, controlled release of drugs or growth factors. The development of such scaffolds is expected to significantly improve outcomes in bone repair and regeneration.

In vivo monitoring technologies are also advancing, with new imaging methods like fluorescence lifetime imaging microscopy (FLIM) and high-resolution MRI offering real-time insights into bone repair processes. These technologies enable continuous assessment of treatment progress and adjustment of therapeutic strategies as needed. Additionally, advancements in nano-mechanical techniques, such as nano-indentation and micropillar compression, are providing more precise measurements of bone mechanical properties, crucial for understanding how bones respond to various forces and stresses.

The future of bone disease management will likely see further ***integration of these emerging technologies in the research and in the clinical practice***, including the development of novel nanomaterials and advanced computational models. Research will continue to explore new ways to enhance drug delivery systems, improve the accuracy of diagnostic tools, and optimize therapeutic approaches. By harnessing these innovations, researchers aim to address the complex challenges associated with bone fragility, offering more effective, personalized, and precise treatments. This comprehensive approach holds the potential to significantly improve patient outcomes, reduce the burden of bone-related diseases, and advance our

understanding of bone health at the most fundamental levels. The ongoing evolution in nano-scale imaging, materials science, and computational modeling promises to unlock new frontiers in bone disease management, ultimately leading to more targeted and effective strategies for preventing and treating bone fragility.

In conclusion, the investigation of bone damage processes at the nano-scale constitutes a fundamental aspect not only for comprehending the origin of fragility fracture mechanisms but also for providing effective and preventive strategies for bone repair. Early diagnosis and an enhanced understanding of bone mechanisms at the nano-scale are imperative to mitigate the health, economic, and societal impact of fragility fractures. Traditional clinical methodologies for assessing bone fragility focus on identifying fractures after their occurrence; in contrast, nano-scale imaging technologies, such as atomic force microscopy and high-resolution electron microscopy, offer the capability to evaluate damage at the sub-micro scale. Simultaneously, advanced nano-mechanical characterization methods, including nanoindentation and micropillar compression, provide precise measurements of mechanical properties, elucidating bone responses to diverse forces. Utilizing advanced computational tools, nano-scale modeling simulations delve into the behavior of bone components under varying conditions, offering crucial insights into fracture mechanisms and failure modes. This comprehensive understanding facilitates the development of preventive strategies by targeting specific nano-scale aspects contributing to bone fragility. Furthermore, the analysis of bone at the nano-scale is indispensable for comprehending mechanisms at higher scales, including the impact of disease, age-related changes, and treatments on fracture processes. While assessing the effect of drugs, the complex nature of characterizing the atomic and structural integrity of bone at the nano-scale remains an experimental challenge. Consequently, the ongoing development of models and techniques capable of predicting fracture risk represents a dynamic field of study.

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T.K. Conceptualization, Investigation, Roles/Writing - original draft, Writing - review & editing

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Declaration of Competing Interest

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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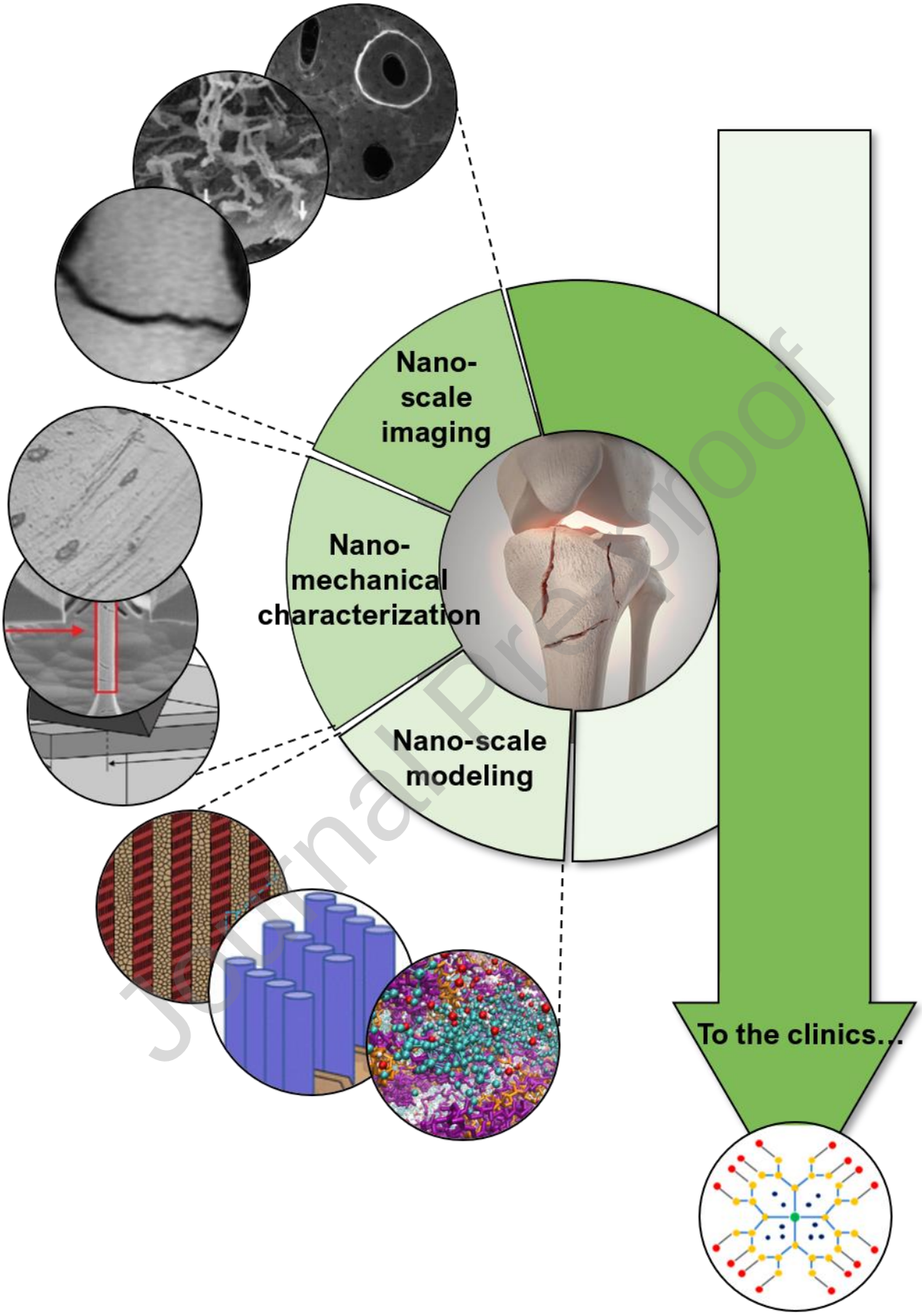

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