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Review Article

An updated overview of the search for biomarkers of osteoporosis based on human proteomics

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ABSTRACT

Osteoporosis is a chronic metabolic disease that increases bone fragility and, leads to severe osteoporotic fractures. In recent years, the use of high-throughput omics to explore physiological and pathological biomarkers related to bone metabolism has gained popularity. In this review, we first briefly review the technical approaches of proteomics. Additionally, we summarize the relevant literature in the last decade to provide a comprehensive overview of advances in human proteomics related to osteoporosis. We describe the specific roles of various proteins related to human bone metabolism, highlighting their potential as biomarkers for risk assessment, early diagnosis and disease course monitoring in osteoporosis. Finally, we outline the main challenges currently faced by human proteomics in the field of osteoporosis and offer suggestions to address these challenges, to inspire the search for novel osteoporosis biomarkers and a foundation for their clinical translation. In conclusion, proteomics is a powerful tool for discovering osteoporosis-related biomarkers, which can not only provide risk assessment, early diagnosis and disease course monitoring, but also reveal the underlying mechanisms of disease and provide key information for personalized treatment.

The translational potential of this article: This review provides an insightful summary of recent human-based studies on osteoporosis-associated proteomics, which can aid the search for novel osteoporosis biomarkers based on human proteomics and the clinical translation of research results.

1. Introduction

Osteoporosis (OP) is a chronic progressive metabolic bone disease characterized by low bone mineral density (BMD) and impaired bone microarchitecture [1]. Clinically, it often manifests as chronic pain and increased susceptibility to low-traumatic fractures, resulting in an impaired quality of life and higher mortality in patients [2,3]. However, most patients with osteoporosis do not exhibit noticeable clinical symptoms. Currently, the most effective approach for diagnosing osteoporosis and evaluating fracture risk is based on BMD measurements using dual-energy X-ray absorptiometry (DXA) [4]. It is difficult to achieve early diagnosis and monitoring. Therefore, there is an urgent need for biomarkers that can predict and diagnose bone metabolic abnormalities in the early stages, and a deeper understanding of the osteoporosis process is required.

Proteomics focuses on studying all proteins present in a genome, cell, or whole organism, and reveals the spatial and temporal variations in protein expression through qualitative and quantitative analysis of protein structure and function [5]. Compared to genes or transcript products, proteins reflect changes in the physiological processes of a disease more intuitively [6,7]. Currently, in human-based osteoporosis research, the assessment of protein expression is primarily focused on the serum or blood cells in the peripheral blood. Many studies have conducted proteomic analyses of human peripheral blood to identify and characterize the proteins associated with osteoporosis [8]. Advances in proteomic technology and the increasing depth of osteoporosis research have led to the development of human bone (bone tissue and bone marrow)-based proteomics. This approach directly reflects the authentic biological environment of skeletal pathology and, offers a new avenue for acquiring more precise and valuable information.

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Biomarkers are biochemical indicators of changes in the structure and function of biological systems at different levels, including systems, organs, tissues, cells, and subcells. They are also closely associated with disease progression [9]. With the development of omics, it has become an indispensable tool in the search for new biomarkers. Studies have shown that proteomics can identify new biomarkers for predicting osteoporosis risk and assessing disease progression in the early stages when bone loss has not yet occurred significantly. At the same time, the underlying mechanisms of osteoporosis disease can be further understood and refined through proteomics [10]. In conclusion, proteomics can be used on a large scale to search for potential new biomarkers as a complement to the DXA, mainly for early prevention, diagnosis and monitoring of osteoporosis.

Currently, a systematic summary of studies exploring osteoporosis biomarkers based on human proteomics is lacking. This review first provides a brief overview of the technical aspects of proteomics. Then, according to the source of protein tissue (Fig. 1), the advancement of human proteomics research related to osteoporosis is systematically reviewed. Finally, the primary obstacles facing human proteomics in the field of osteoporosis are discussed, and suggestions to solve the current challenges are proposed. This review aimed to provide ideas for the search for novel biomarkers for osteoporosis and to lay the groundwork for their clinical translation.

2. Overview of proteomics research

As research progressed, it became evident that living organisms are complex systems, and that a comprehensive understanding of life processes and disease mechanisms cannot be achieved solely by studying individual biomolecules. Systematic research is essential for gaining a deeper insight into life. Thus, the concept of “omics” emerged, which focuses on the collective characterization and quantitative study of a particular class of macromolecules in an organism, aiming to reveal the functioning of life at the systemic level [11]. As the ultimate executors of life activities, proteins operate beneath the central dogma and play a crucial role in the development of both health and illness [12]. Proteomics has evolved to investigate the biological functions of proteins in this context [13].

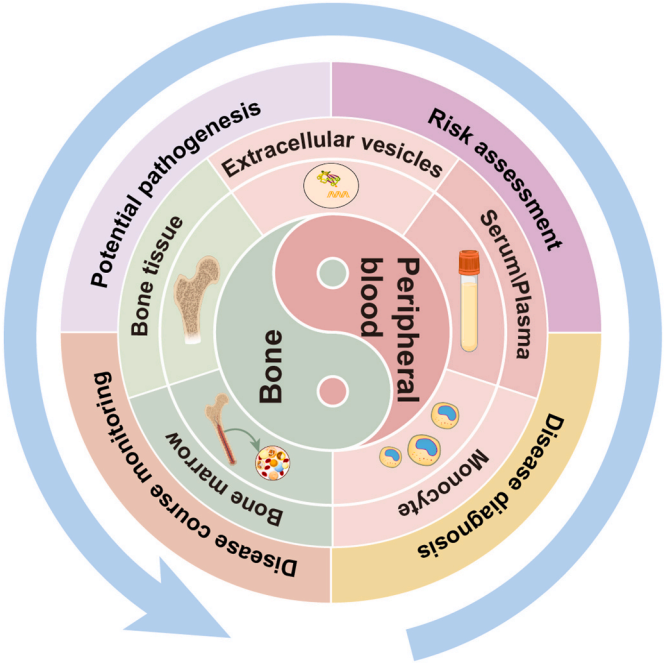

Fig. 1. Schematic illustration of human proteomics sample sources. Figure created with the help of https://www.biorender.com.

Proteomics belongs to the scientific field focused on studying the composition, structure, and function of proteins in living organisms. A typical process involves (i) Sample collection. (ii) Protein extraction. (iii) Separation and enrichment. (iv) Proteomics profiling. (v) Data analysis. (vi) Functional studies (Fig. 2) [14,15].

Early techniques for targeting proteins are low-throughput, including enzyme-linked immunosorbent assay (ELISA) and western blotting, which rely on antibodies against specific epitopes of proteins to identify and quantify their expression levels. In the 1970s, gel electrophoresis (GE) techniques were developed for separating complex protein samples, marking the beginning of the systematic analyses of multiple proteins. GE typically utilizes protein size or isoelectric points for protein separation and is a widely used analytical method in proteomic research. However, gel-based techniques are often time-consuming and unsuitable for high-throughput proteomic analysis [16]. Various technologies have been developed rapidly in recent years. Among these, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has made proteomic analysis and identification simple and rapid. LC-MS/MS combines the high separation power of liquid chromatography with mass spectrometry to provide structural information and improve the sensitivity and selectivity of detection [17]. This technology is crucial for advancing proteomic research (Fig. 3).

Advancements in proteomics have provided a systematic tool for life science research and have helped overcome the limitations of genomics. This integrated approach contributes to searching for specific biomarkers and revealing the mechanisms underlying disease development, thereby promoting a deeper understanding of biological phenomena and disease mechanisms [19].

3. Human proteomics based on peripheral blood

In recent years, proteomic techniques have been extensively applied in the research of osteoporosis. Proteomics research has shown a substantial correlation between proteomics-based biomarkers and osteoporosis [20]. Peripheral blood is a clinically accessible sample obtained through minimally invasive means and is well-suited for assessing human health and disease states in most biological and clinical applications performed on large populations. Among the various studies included in this review, peripheral blood samples were selected as the primary source for the proteomic analysis. The scarcity of studies on bone tissue samples may be attributed to the difficulty of obtaining these samples and the complexity of the procedure. Peripheral blood samples, including serum, plasma, monocytes, and extracellular vesicles (EVs), are all tightly associated with proteins and overlap with each other.

3.1. Monocyte-based proteomics

Monocytes are leukocytes derived from bone marrow hematopoietic stem cells. As the progenitors of osteoclasts, monocytes play an important role in bone remodeling [21,22]. The journey from peripheral monocytes to osteoclasts involves three early stages: chemotaxis towards the site of osteoclast formation, adhesion to endothelial cells in the vicinity of the bone, and transendothelial migration into the bone microenvironment [23]. It is currently challenging to obtain fresh and sufficient osteoclasts from human bone tissue for research purposes. Peripheral blood monocytes (PBMs) are primarily selected for osteoporosis research due to their accessibility and crucial role in osteoclastogenesis, athough they do not accurately reflect the properties of osteoclasts [24]. Proteomics based on PBMs refers to the proteomic analysis of monocytes isolated from the peripheral blood to identify biomarkers associated with osteoporosis (Table 1).

From 1000 Chinese premenopausal women, Deng et al. screened 30 participants with extremely high and extremely low hip BMD, collected and isolated PBMs, and finally obtained 38 differentially expressed proteins via MALDI-TOF/TOF-MS. They found that the expression of gelsolin (GSN) was up-regulated in patients with extremely low BMD

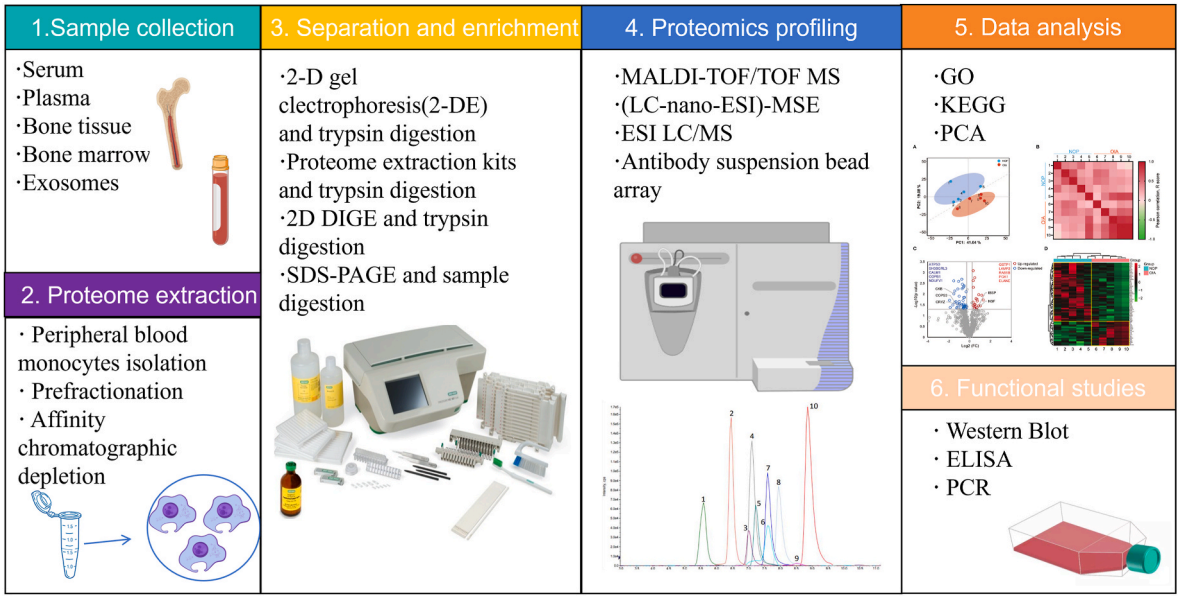

Fig. 2. Schematic representation of the workflow for the proteomic approach in osteoporosis. Figure created with the help of https://www.biorender.com.

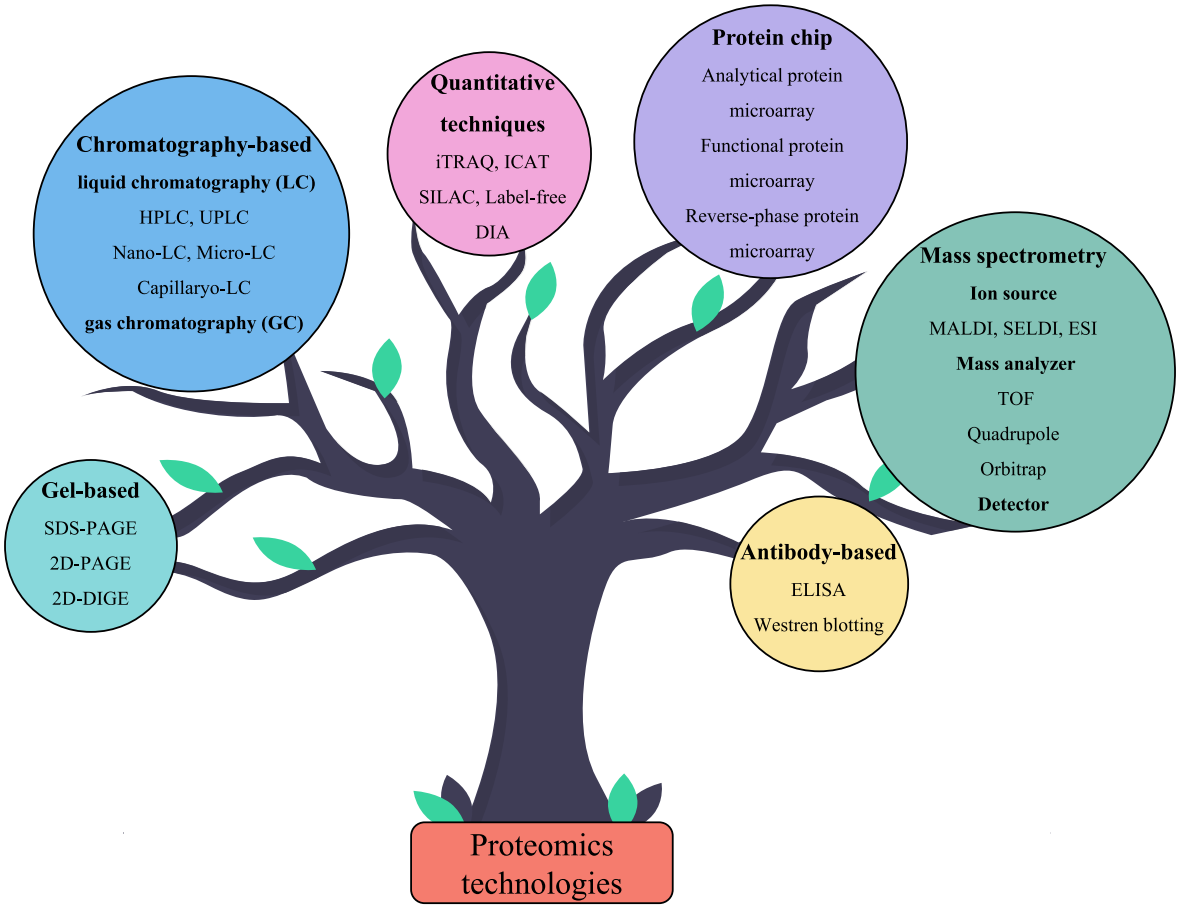

Fig. 3. An overview of proteomics techniques. Figure created with the help of https://www.biorender.com.

[25]. In terms of PBMs, GSN is also the most frequently reported protein. Researches have demonstrated that GSN is involved in the regulation of actin polymerization/depolymerization and the cytoplasmic gel state, which in turn affects cell migration and adhesion [32,33]. In osteoclasts, GSN plays a critical role in podosome assembly, rapid cell movements, and signal transduction through the α vβ3 integrin. A GSN-deficient mouse model showed that osteoclasts present an abnormal actin cytoskeletal architecture and reduced rates of osteoclast motility, contributing to reduced bone resorption in vivo and blocking podosome-associated signal transduction [34]. Following integrin

Table 1
Summary of human proteomics based on peripheral blood monocytes.

Reference	Subjects	Number enrolled (Case/ Control)	Aged	Cell/Tissue	Proteomic Profiling Method	Key find
Deng et al. [25]	Premenopausal Chinese women	30 (15/15)	20–45	PBMs	MALDI-TOF/ TOF-MS	38 differentially expressed proteins found, among which RSU1, GSN, SOD2 upregulated in low BMD group; GPX1, P4HB down regulated in low BMD group.
Deng et al. [26]	Postmenopausal Caucasian women	28 (14/14)	Post-menopause	PBMs	LC-nano-MS	Up-regulation (twofold) of ANXA2 in low BMD group; results confirmed at mRNA and DNA level.
Deng et al. [21]	Premenopausal Caucasian women	34 (17/17)	Pre-menopause	PBMs	LC-nano-ESI-MS	57 differentially expressed proteins, among which GSN was down-regulated in the low BMD group.
Daswani et al. [27]	Pre and post-menopausal Indian women	40 (20/20)	30-40、50–60	PBMs	LC-MS/MS	45 differentially expressed proteins, with HSP27 distinctly upregulated in the low BMD.
Zhang et al. [28]	Postmenopausal Caucasian women	42 (21/21)	Post-menopause	PBMs	LC-nano-ESI-MS	Up-regulation of LMNB1, ANXA2P2, ANXA2 in low BMD group. Down-regulation of OC654188, PPIA, TAGLN2, YWHAB in low BMD group.
Zeng et al. [22]	Premenopausal Caucasian women	33 (17/16)	Pre-menopause	PBMs	UPLC-nano-ESI-MS/MS	30 differentially expressed proteins, among which RHOA, ITGA2B, and GSN significantly upregulated in the high BMD group.
Zhu et al. [29]	Caucasian men	59 (29/30)	Pre-menopause、Post-menopause	PBMs	LC-nano-ESI-MS	16 significant and 22 suggested DEPs, among which ALDOA, MY-H14, and RAP1B were up-regulated in the low BMD group.
Zeng et al. [30]	Caucasian men	59 (29/30)	Pre-menopause、Post-menopause	PBMs (membrane)	2D-nanoLC-ESI-MS/MS	36 differentially expressed proteins, among which down-regulation of ITGB1 and ACTN1 in the high BMD group and up-regulation of P4HB and D36 in the high BMD group.
Zeng et al. [31]	Caucasian female	76	Pre-menopause、Post-menopause	PBMs	LC-MS/MS	Developed the first comprehensive proteome knowledge base specifically for human monocytes, and a total of 2237 unique monocyte-expressed genes were cataloged.

activation, GSN connects osteoclasts to the bone matrix during bone resorption and regulates the formation of osteoclast actin rings [35]. To sum up, GSN decreases bone mass by stimulating osteoclast migration, adhesion, and activity, which promotes osteoclastogenesis and bone resorption. Using the LC-nano-ESI-MS technique, Zhou et al. found that GSN expression in PBMs from white men increased in patients with low BMD [29]. Intriguingly, some researchers have reported that GSN expression is up-regulated in patients with high BMD in PBMs from premenopausal white women via LC-MS/MS [21,22]. Racial differences, sex, and technological differences may account for these contradictory results. According to a recent study, GSN affects androgen receptor (AR) and enhances androgen-induced AR trans-activation, which can inhibit monocyte proliferation, promote apoptosis, and ultimately prevent bone resorption [36].

Deng et al. observed that superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPX1) were significantly up-regulated and down-regulated in the osteoporosis group, respectively [25]. SOD2 catalyzes the production of hydrogen peroxide (H_2O_2) from superoxide. GPX1, a crucial antioxidant enzyme in the human body, plays a vital role in oxidative stress induced by H_2O_2. Therefore, SOD2 and GPX1 collectively regulate H_2O_2 levels at the cellular level [37]. Previous studies have shown that H_2O_2 has several effects, including promotion of osteoclast differentiation and formation, RANKL expression in the human osteoblast-like MG 63 cell line, and enhancement of the activity of mature osteoclasts in the mouse skullcap [38–40]. Additionally, over-expression of GPX in cells may abolish differentiation and osteoclast formation [41]. Overall, up-regulation of SOD2 and down-regulation of GPX1 promote osteoclast differentiation, formation, and activity, resulting in bone mass loss. Previous studies have shown that women with postmenopausal osteoporosis have significantly higher plasma SOD enzyme activity than controls, and that osteoporotic males exhibit a negative correlation between serum SOD and lumbar BMD [40,42]. This evidence suggests that SOD2 and GPX1 may play an important role in the progression of osteoporosis, possessing the potential to reflect the risk of disease progression and in monitor disease status.

Annexin A2 (ANXA2) is another protein frequently mentioned in several studies on PBMs. Using LC-MS/MS techniques, multiple studies on postmenopausal white and menopausal Indian women have reported that ANXA2 is significantly upregulated in patients with low BMD [26–28]. The study demonstrates a strong association between ANXA and osteoporosis physiopathology. First, increased expression of ANXA2 protein on PBMs, probably by promoting their matrix-penetrating activity, contributes to PBM migration from the blood to sites of bone resorption. Second, ANXA2 is released after PBM reaches bone resorption sites, elevating the local extracellular ANXA2 concentration. Elevated extracellular ANXA2 concentration at bone resorption sites further attract PBMs to these sites by enhancing trans-endothelial migration, resulting in an expanded pool of osteoclast precursors at the sites of bone resorption. Moreover, ANXA2 also promotes monocyte differentiation, osteoclast formation, and bone resorption [43–47]. The role of Anxa2 in bone remodeling is not limited to osteoclastogenesis. High doses of ANXA2 inhibit osteoblast growth [48]. This ultimately leads to bone loss. In the same study, Daswani et al. found that heat shock protein 27 (HSP27), which is related to the RANKL pathway, was significantly upregulated in menopausal Indian women with low BMD, independent of the menopausal status [27]. Recombinant phosphorylated HSP27 boosts monocyte migration in a dose-dependent manner and has a cumulative effect in combination with the chemoattractant effect of recombinant RANKL (rRANKL), eventually resulting in bone loss. In conclusion, HSP27, as molecular chaperone involved in various biological processes, play an important biological function in osteoporosis. HSP27 has the potential to be a diagnostic markers for osteoporosis and is unaffected by female menopause.

Using MAL-DI-TOF/TOF-MS and LC-nano-ESI-MS techniques, studies involving premenopausal Chinese women and White men revealed that prolyl 4-hydroxylase β subunit (P4HB) was down-regulated in patients with low BMD compared to those with high BMD, in both genders [25,29,30]. Meanwhile, a study on the pathogenesis of osteoporosis showed that P4HB was down-regulated in osteoblasts of osteoporosis patients [49]. P4HB can assist in protein assembly and folding, and participates in the regulation of apoptosis. However, the exact mechanism of how P4HB leads to bone loss has not yet been elucidated. This may provide a potential therapeutic target that is independent of race. In 2017, Zeng et al. created the first comprehensive proteomic database exclusively for human monocytes based on LC-MS/MS technology, which included 2237 unique genes expressed in

human monocytes. This database serves as a valuable reference for future omics studies on peripheral monocytes [31].

In summary, the migration of monocytes into the bone microenvironment is essential for osteoclastogenesis, which leads to bone loss and is synergistically regulated by various molecules. Therefore, proteomics research provides an opportunity to simultaneously explore changes in a large number of protein molecules in monocytes, which greatly facilitates the discovery of biomarkers for osteoporosis in monocytes.

3.2. Serum/plasma-based proteomics

Serum/plasma proteomics refers to the analysis of plasma or serum to identify biomarkers. Zhou et al. identified a negative association between plasma levels of ANXA2 and BMD in a group of older Chinese individuals using LC-MS/MS [50]. This association trend was consistent with that reported for PBM and was further validated in the population by ELISA. Wang et al. performed a study on a subset of 164 elderly Chinese patients from a larger group of 6000 individuals, using ELISA to quantify plasma GSN levels. The findings demonstrated a significant differential expression of plasma GSN between participants with extremely low and extremely high hip BMD. There was a negative correlation between plasma GSN and BMD, particularly in older women [51]. These studies suggest that ANXA2 and GSN are more reliable potential diagnostic biomarker for osteoporosis.

Bhattacharyya et al. discovered that the inter-alpha trypsin inhibitor heavy chain H4 (ITIH4) is linked to reduced bone mass in postmenopausal women using protein chip SELDI TOF-MS technology. ITIH4 may be a circulating product of increased bone resorption, reflecting increased osteoclast activity associated with elevated bone turnover. The discovery of ITIH 4 provides a novel approach to assessing the condition of high bone turnover. Further research is needed to assess the expression of ITIH4 in bone and whether it is stored in the bone matrix and is a substrate for enzymatic degradation by osteoclasts [52]. In contrast to numerous cross-sectional studies, Arasu et al. conducted the first prospective cohort study in 2012, which enrolled a total of 9704 older women (aged >65 years), of whom 455 were tested for serum sclerostin by ELISA. The results demonstrated a correlation between elevated serum sclerostin levels and an increased risk of hip fracture. This study identified a promising target for preventing osteoporotic fracture [53]. Sclerostin inhibits osteoblast differentiation by binding to its receptors low-density lipoprotein receptor-related protein 5 and low-density lipoprotein receptor-related protein 6, thus impeding downstream activation of canonical Wnt signaling [54–56]. Moreover, it inhibits the signaling effects of bone morphogenetic protein (BMP) [57]. In addition to affecting osteoblast differentiation and apoptosis, sclerostin stimulates osteoclastogenesis [58]. The Food and Drug Administration (FDA) approved the sclerostin antibody (Romosozumab) in 2019 for the treatment of postmenopausal osteoporosis in individuals at a significant risk of fracture. Martinez-Aguilar et al. used MALDI-TOF/TOF MS proteomic techniques to identify a notable reduction in the levels of vitamin D-binding protein (VDBP) in the blood of postmenopausal women with low BMD and individuals with osteoporotic fractures [59]. VDBP functions as a transporter of vitamin D, delivering it to certain tissues; therefore, it plays a vital role in regulating calcium balance [60]. VDBP deficiency occurs before changes in bone mass, so measuring blood levels of VDBP has the potential to be a biomarker for early detection of osteoporosis risk and evaluation of vitamin D therapy.

The effective execution of omics studies relies significantly on adequate sample size, as larger sample sizes produce more reliable findings [61]. In 2017, Nielson et al. conducted a cross-sectional investigation in the field of osteoporosis proteomics. Their study employed a comprehensive proteomic technique to analyze huge samples and attempted to uncover clinically relevant bone biomarkers in blood [62]. The study utilized LC-IMS-MS proteomics and included a sample of 1874 older males. It discovered that there is a correlation between the 20 proteins identified in the serum and a faster decline in BMD. Of these proteins, five (CD14, CHL1, CO7, FCGBP and PZP) were linked to hip fracture events, whereas the remaining proteins were involved in cellular senescence, complement activation, and innate immunity. This study provides important information for future research in bone biology and fracture prediction.

Prospective studies are better suited for assessing the long-term effects of risk variables on health. In a recent extensive prospective investigation, LC-MS/MS was used to identify 53 proteins associated with osteoporosis in the serum of 3415 Chinese patients [18]. The study utilized machine learning and meta-analysis to construct protein risk scores and the biological age of bones to assess early osteoporosis risk and monitor skeletal aging progression (Fig. 4).

Osteoporosis is a systemic metabolic bone disease closely linked to the immune system [64,65]. Al-Ansari et al. utilized nano LC-MS/MS proteomics technology in an aged population study and discovered that most bone-associated proteins were linked to the immune system (immune globulin and complement). Their work enhances our understanding of the interaction between the skeletal and immune systems, leading to the development of an interdisciplinary area known as osteoimmunology [66]. The term “immunoporosis” was proposed in 2018 by Srivastava et al. to highlight the significant contribution of immune cells in the progression of osteoporosis [67]. Immunological osteoporosis offers a valuable perspective for understanding the intricate interplay between the immune and skeletal systems. This allows for the use of immunological knowledge to clarify the disease processes that cause osteoporosis, potentially yielding valuable insights for developing novel therapies and pharmaceuticals targeting osteoporosis in the future.

In summary, serum/plasma is commonly used to evaluate patients with different disorders and is prominent in clinical proteomics research. Although peripheral blood samples are susceptible to contamination from various disorders, they are the preferred choice for osteoporosis proteomics research owing to their high protein content, minimal invasiveness, and high reproducibility (Table 2).

3.3. EVs-based proteomics

Peripheral blood EVs proteomics refers to the proteomic analysis of EVs isolated from peripheral blood to identify relevant biomarkers. Peripheral blood EVs are heterogeneous and consist of exosomes and microvesicles of different sizes, origins, and antigenic makeup. Exosomes are derived from multivesicular bodies (MVBs) and range from 40 to 100 nm in size. They are secreted from cells through the fusion of MVBs with the plasma membrane. Microvesicles are heterogeneous membrane vesicles up to 1 μm in size, derived directly from the cell membrane of activated cells by disrupting the cortical cytoskeleton. Numerous cells, including cancer cells, immune cells, platelets, mesenchymal stem cells, osteoblasts, and osteoclasts, release EVs [71]. EVs can either directly stimulate target cells through receptor-mediated interactions or transfer various biological components to target cells, including ligands, receptors, proteins, DNA, mRNA, microRNAs, and intact organelles (e.g. mitochondria). EVs participate in diverse biological processes, and research has demonstrated that the development of osteoporosis is significantly influenced by EVs [72,73].

Using nano-HPLC-ESI-MS/MS proteomics techniques, Pepe et al. found that postmenopausal Caucasian women with osteoporosis exhibited elevated plasma synthesis and release of EVs compared to normal individuals. In patients with osteopenia, the number of circulating EVs does not vary; however, there are alterations in the protein concentrations within the EVs [73]. Thus, EVs may be a good source of osteoporosis biomarkers.

A study utilizing LC-MS/MS proteomic techniques examined the differences in protein expression between older patients with osteoporosis, osteopenia, and those with normal BMD. The analysis revealed a significant decrease in the levels of integrin-associated proteins in

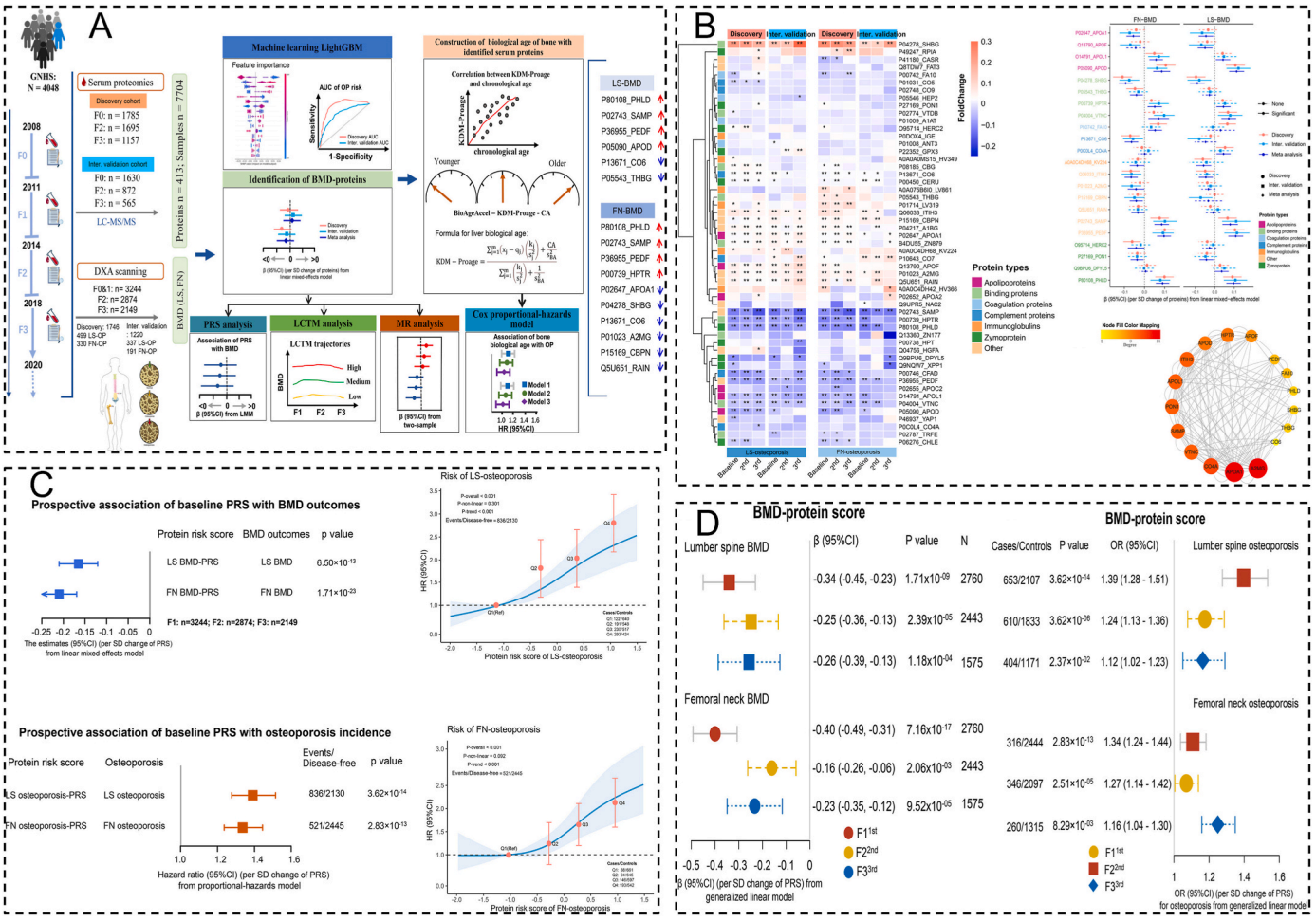

Fig. 4. Proteome-wide profiling reveals dysregulated molecular features and accelerated aging in osteoporosis by prospective study (A) Overview of the study cohort and design. (B) Prospective associations between serum proteins with osteoporosis and BMD. (C) Prospective associations of PRS with osteoporosis and BMD. (D) The prospective associations between biological age scores and osteoporosis risk [18]. Copyright 2023, John Wiley and Sons.

osteoporosis patients, specifically β1 and β3 integrins and CD34, which hindered the ability of integrins to sense and activate osteoclasts. Differential exosomal proteins in the osteopenia group promoted both osteoclast activation and new bone formation, resulting in a compensatory increase in bone remodeling. Exosomal differential proteins in the aged normal group protect bone health by promoting osteoblast adhesion and inhibiting aging-associated oxidative stress [74]. In conclusion, the progression of osteoporosis can be monitored by changes in EVs present in the plasma.

In other similar studies, Chen et al. employed LC-nano-MS/MS proteomics to detect 45 differentially expressed proteins in an older Chinese population with hip fractures. Among these, four proteins-proteasome subunit beta type-9 (PSMB9), alanine-tRNA ligase, cytoplasmic (AARS), poly(rC)-binding protein 2 (PCBP2), and V-type immunoglobulin domain-containing suppressor of T-cell activation (VSIR)-were found to be closely associated with osteoporosis [75]. PSMB9 is a protease complex that promotes bone formation by enhancing the activity and number of osteoblasts [76]. Qin et al. suggested that bortezomib, the first proteasome inhibitor approved by the FDA for the treatment of multiple myeloma, plays an important role in the treatment of radiation-induced osteoporosis [77]. There is no evidence linking the other three proteins to osteoporosis. However, PCBP2 has been demonstrated to interact with nuclear heterogeneous nuclear ribonucleoprotein K (HNRPK), which may be involved in osteoclast formation and regulation of bone homeostasis [78]. Additionally, VSIR is an immunomodulatory receptor that inhibits BMP4 signaling and is involved in the formation of bones and cartilage, particularly tooth and limb development and fracture repair [79]. AARS has been implicated as a contributing factor in muscle weakening and atrophy [80]. The study indicates that the exosomal protein index consisting of these four proteins achieves an area under the curve (AUC) of 0.805 for the classification of osteoporosis and has the potential to be a diagnostic biomarker for osteoporosis. Although their diagnostic efficacy is poor, they can play a role in early risk assessment of osteoporosis as an adjunct diagnosis to DXA.

In terms of microvesicles, Huo et al. discovered that among 84 patients, 19 proteins were up-regulated and 5 proteins were down-regulated in the low BMD group compared to the control group using LC-MS/MS technology. Among the up-regulated proteins, changes in serum Vinculin, Filamin A, and Profilin 1 levels were further verified [69]. These proteins are all associated with bone homeostasis and strongly interact with each other. Vinculin and Filamin A are important osteoclast regulators. Vinculin is a ubiquitously expressed actin-binding protein located on the cytoplasmic surface of integrin-containing podosomes in osteoclasts and is closely related to two key regulators of the osteoclast cytoskeleton, Talin1 and Arp2/3 [81,82]. Filamin A is a ubiquitous actin-binding protein and a necessary regulator of podosome and sealing zone formation in osteoclasts [83]. Profilin 1 is closely related to BMPs, which play a vital role in bone formation by controlling osteoblast differentiation and migration [84]. Profilin 1 was further pre-validated in an independent sample set, which could differentiate osteoporosis from other diseases with good sensitivity [85]. We suggest

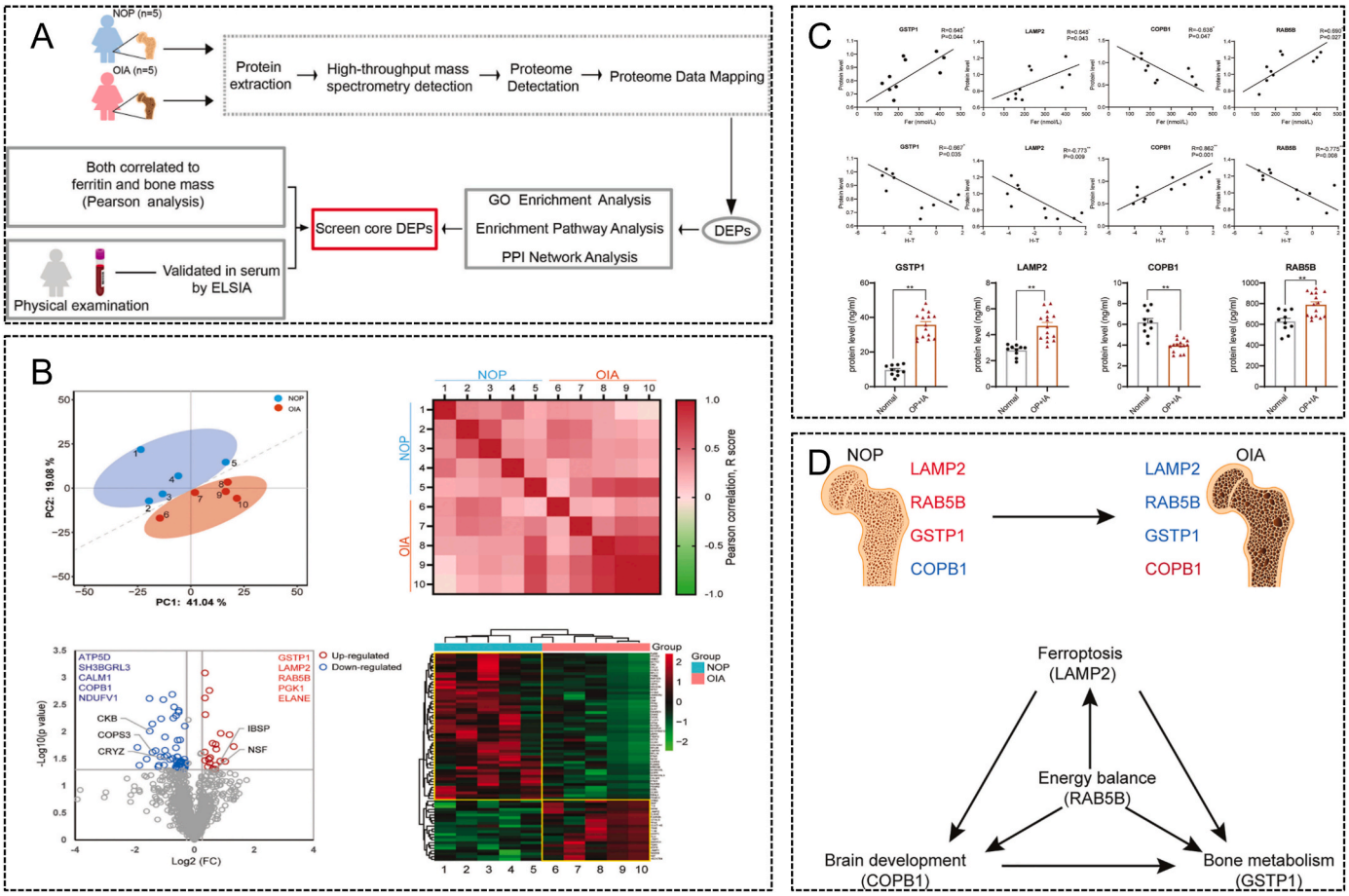

Fig. 5. Human bone tissue and serum-based screening for biomarkers of osteoporosis with iron accumulation (A) Workflow for quantitative mass spectrometry profiling. (B) Quantitative proteomics analysis of human bone from osteoporosis patients with iron accumulation. $^{**}P < 0.01$. (C) Serum protein levels of physical exam volunteers were tested by ELISA kits. (D) Graphed summary of four core proteins [63]. Copyright 2022, Frontiers.

Table 2
Summary of human proteomics based on plasma/serum.

Reference	Subjects	Number enrolled (Case/ Control)	Aged	Cell/ Tissue	Proteomic Profiling Method	Key find
Bhattacharyya et al. [52]	Postmenopausal women	58 (30/28)	60–88	Serum	Protein chip SELDI TOF-MS	Down-regulation of ITIH4 peptide fragments in the high bone turnover group; result confirmed at an additional 59 postmenopausal women.
Qundos et al. [68]	Postmenopausal women	22 (16/6)	Post-menopause	Plasma	Antibody suspension bead arrays	Autocrine motility factor receptor (AMFR) was lower in osteoporosis patients.
Nielson et al. [62]	Non-Hispanic white men	1874 (453/ 1184/237)	> 65	Serum	LC-IMS-MS	20 proteins were associated with accelerated BMD loss. Five proteins were associated with incident hip fracture (CD14, CHL1, CO7, B2MG, FCGBP).
Zhou et al. [50]	Chinese Han elderly	87 (45/42)	> 65	Plasma	LC-MS/MS	Anxa2 protein could attenuate osteoblast growth and be associated with hip BMD and Osteoporotic fracture in the Chinese elderly.
Martínez-Aguilar et al. [59]	Menopausal Mexican women	30 (10/10/10)	≥45	Serum	MALDI TOF/TOF MS	27 proteins were identified, among which VDBP correlated with low BMD (osteopenia and osteoporosis).
Zhou et al. [69]	Chinese postmenopausal women	84 (20/32/32)	> 65	Plasma	LC-MS/MS	Significantly down-regulated of Abl Interactor 1(ABI1) in osteoporosis and osteoporotic fracture.
Huang et al. [70]	Postmenopausal women	54 (18/18/18)	Post-menopause	Plasma	LC-MS/MS	The expression of lysozyme C (P61626) was negatively related to BMD, and the expression of glucosidase (A0A024R592) and disulfide isomerase A5 (Q14554) was positively related to BMD.
Al-Ansari et al. [66]	Old people	69 (22/22/25)	> 50	Plasma	Nano LC-MS/MS	20 differentially expressed proteins, levels of eight proteins were upregulated in osteopenia group, and the levels of eleven proteins were downregulated in the osteopenia group.
Xu et al. [18]	Chinese	3415 (1785/ 1630)	45–75	Serum	LC-MS/MS	53 proteins associated with osteoporosis were identified, including PHLD, SAMP, PEDF, HPTR, APOA1, SHBG, CO6, A2MG, CBPN, RAIN APOD, and THBG.

that Profilin 1 has potential utility in the future molecular diagnosis of osteoporosis as an adjunctive screening prior to DXA.

In summary, the complex process of extracting and isolating EVs may affect detection speed. However, the proteins found in these vesicles can be transferred between organs via bodily fluids and other pathways. This transfer offers a more precise indication of the physiological or pathological conditions of different tissues. With technological advancements, the ability to detect and identify proteins in EVs has improved in terms of sensitivity and specificity. Consequently, the proteomic analysis of proteins in EVs is regarded as a vital component of osteoporosis biomarker research (Table 3).

4. Human proteomics based on bone

4.1. Bone tissue-based proteomics

Bone tissue proteomics refers to the identification of relevant biological markers directly from bone tissue using proteomic techniques. Bone tissue primarily comprises inorganic mineral materials (mainly hydroxyapatite) and organic materials derived from the intracellular and extracellular matrices [86]. Typically, bone tissue samples are obtained during surgery and this invasive method is mostly employed for scientific research rather than routine clinical situations. In proteomics studies, the primary constraints of this method are the limited number of samples and the challenges of access. Despite these limitations, analyzing protein expression in a true biological environment that accurately reflects bone histopathology can provide more precise and valuable information.

Based on LC-MS/MS proteomics techniques, Alves et al. constructed a human bone trabecular proteomic library targeting non-collagenous proteins (NCPs) by analyzing femur samples from two healthy older men and two healthy older women. The library involves 1051 NCPs, including most of the classical bone matrix proteins identified [86]. Although NCPs account for only 10 % of total bone protein content, this study provides an informative library of bone proteins that could serve as a source of novel bone formation modulators as well as osteoporosis-related biomarkers. Chaput et al. conducted a proteomic analysis using LC-MS/MS on the femoral necks of osteoarthritis patients. They aimed to evaluate the expression of bone tissue proteins between individuals with osteopenia and normal BMD. It was found that carbonic anhydrase I (CA1) and phosphoglycerate kinase 1 (PGK1) levels were significantly increased, whereas apolipoprotein A-I (apoA-1) levels were significantly decreased in patients with osteopenia. Most significantly, the aforementioned differential proteins did not exhibit substantial variations in blood samples, indicating that alterations in protein levels in bone tissue may offer a more precise indication of changes in the bone [87].

Iron accumulation has been identified as an independent risk factor for osteoporosis and can significantly accelerate bone loss in osteoporosis patients, particularly in postmenopausal women [88,89]. Understanding the role of iron in bone metabolic disorders is crucial for osteoporosis research. For the first time, our group utilized LC-MS/MS proteomics technology to investigate alterations in osteoporosis-related proteins in human bone affected by iron accumulation. Ultimately, four core proteins (GSTP1, LAMP2, COPB1, and RAB5B) were significantly differentially expressed in the bone tissues of 10 postmenopausal patients who underwent hip arthroplasty. Furthermore, this study confirmed the alterations in the concentrations of the respective proteins in the serum of patients undergoing medical examination. GSTP1 plays a role in bone remodeling and catalyzes the detoxification of endogenous and exogenous substances by binding to glutathione (GSH). Iron can induce intracellular changes in the GSH/GST antioxidant system [90]. LAMP2 deficiency reduces the cytosolic cysteine concentration, resulting in low GSH, poor antioxidant capacity and mitochondrial lipid peroxidation, ultimately leading to ferroptosis [91]. Depletion of COPB1 in cells resulted in decreased cell survival, impaired autophagy, and ER stress [92]. RAB5B plays a role in cell migration and proliferation [93]. However, there are no studies on LAMP2, COPB1 or RAB5B concentrating on bone metabolism-related diseases, so our team is working to improve this area of research. In summary, we hypothesize that these critical proteins are involved in the occurrence of osteoporosis with iron accumulation by affecting the interaction of ferroptosis, energy metabolism, brain development, and bone metabolism. These findings provide novel insights for further investigations of the mechanisms underlying osteoporosis and iron accumulation, screening biomarkers, and therapeutic targets (Fig. 5) [63].

Undoubtedly, bone tissue proteomics offers distinct benefits in the elucidation of bone disorders. Although acquiring bone tissue samples may be challenging and invasive, physiological and pathological changes in bone can be more accurately reflected by directly analyzing the proteins in the bone tissue. Given that bone tissue proteomics can offer more bone-specific information, it is more likely to find key molecules related to bone disease compared to serum proteomics. Investigating the alterations in core proteins in the bones of patients with osteoporosis is essential for deciphering the pathogenesis of the disease and finding new biomarkers. This focused and precise method offers a valuable understanding of the causes and features of bone lesions, thereby providing robust evidence for the advancement of more efficient treatments. Although there are certain technical difficulties, bone tissue omics continues to be a potent method for exploring bone metabolism.

Table 3
Summary of human proteomics based on extracellular vesicles.

Reference	Subjects	Number enrolled (Case/ Control)	Aged	Cell/Tissue	Proteomic Profiling Method	Key find
Xie et al. [74]	Osteoporosis, osteopenia, elderly normal volunteers, youth normal volunteers	139 (31/46/ 26/36)	21–84	Serum-derived exosomes	LC-MS/MS	1371 proteins were identified. Different proteins play different roles in osteoporosis, osteopenia, and normal groups
Huo et al. [85]	Osteopenia, osteoporosis, normal patients	84 (28/28/ 28)	> 50	Serum MVs	LC-MS/MS	200 proteins were identified, among which 19 proteins were upregulated and five proteins were downregulated in the osteopenia group and osteoporosis group.
Chen et al. [75]	Osteopenia, osteoporosis, normal Chinese	60 (30/20/ 10)	Post-menopause	Serum-derived exosomes	LC-nano-MS/ MS	Forty-five differentially expressed proteins were identified and PSMB9, AARS, PCBP2, and VSIR were further verified in a validation set.
Pepe et al. [73]	Osteoporotic, osteopenic, normal postmenopausal Caucasian women	146 (54/48/ 44)	50–85	Serum-derived EVs	Nano-HPLC-ESI-MS/MS	Increased production and release in the plasma of EVs in osteoporosis, the EV protein content was increased in osteoporosis and osteopenia, and no significant alterations of EV protein quantification were detected between osteoporosis and osteopenia.

4.2. Bone marrow-based proteomics

Bone marrow proteomics refers to the identification of relevant biological markers directly from the bone marrow through proteomics techniques. The bone marrow, which is present in the cancellous lacuna of the bone and the marrow cavity of the long bone, is a soft and blood-rich tissue composed of various types of cells and reticular connective tissue. The bone marrow can be classified as red bone or yellow bone marrow based on its distinct structures. Soluble factors produced by cells in the bone marrow for intercellular communication constitute the bone marrow microenvironment [94]. The bone marrow microenvironment is the most important site for bone remodeling and is indispensable for maintaining bone homeostasis.

Zhou et al. collected bone marrow supernatants of vertebral from eight Chinese patients undergoing posterior lumbar interbody fusion and identified 172 up-regulated and 47 down-regulated proteins through LC-MS/MS proteomics [95]. These proteins play multiple biological roles in osteoblast differentiation, lipid metabolism, and cell migration, forming an intricate network of protein–protein interactions. Researchers have identified five key regulatory mechanisms that contribute to the development of osteoporosis: splicing, translation, protein degradation, cytoskeletal organization, and lipid metabolism. Among them, protein factors such as DEAD box protein 5 (DDX5), 26S protease regulatory subunit 7 (PSMC 2), casein kinase I isoform alpha (CSNK1A1), integrin-linked kinase (ILK), and tropomyosin alpha 4 (TPM4) exhibit altered expression in the bone marrow microenvironment of osteoporosis patients. DDX5 is a key protein in the spliceosome-associated cluster that inhibits osteogenic differentiation of mesenchymal progenitor cells by participating in the transcription of RUNX2 [96]. PSMC2 and CSNK1A1 are proteasome-associated proteins. PSMC2 is involved in the regulation of RUNX2 through proteasome-mediated ubiquitin-dependent degradation [97]. CSNK1A1 is a component of the β-catenin destruction complex which regulates canonical Wnt signaling. ILK and TPM4 are cytoskeleton-associated proteins that affect the migration and adhesion of osteoblasts and osteoclasts, thereby exerting an integrative influence on both bone formation and resorption. This discovery presents a novel avenue for finding biomarkers for osteoporosis.

The study of the bone marrow microenvironment is challenging because of its anatomical complexity and technical difficulties. To date, limited research exists on proteomics using bone marrow in the field of osteoporosis. However, compared to the peripheral blood, the molecules in bone marrow supernatant can more accurately, more directly reflect the bone marrow microenvironment of partial physiological and pathological state. It is also the best source of osteoporosis biomarkers. Furthermore, it provides a new perspective for the study of bone marrow changes and potential molecular mechanisms of osteoporosis. Despite existing challenges, the utilization of proteomics technology in evaluating the bone marrow microenvironment is expected to offer valuable tools and insights for advancing research on bone health and disease [98].

5. Challenges and future directions

Proteomics has been extensively used in the field of bone physiology and pathology to identify prospective biomarkers and therapeutic targets, particularly for osteoporosis. The field of osteoporosis research can greatly benefit from proteomics. Proteomics can be utilized not only to identify individuals at high risk of osteoporosis and osteoporotic fractures, but also to discover novel targets and approaches for the prevention and treatment of osteoporosis.

This review focuses on human proteomics, which is currently facing major challenges in the field of osteoporosis. (i) Insufficient coverage and sensitivity: Given the current developments in proteomic technology, some low-abundance peptides/proteins and post-modified proteins remain undetectable when exploring protein biomarkers [99]. (ii) Limitation of sample source and quantity: There is limited omics research based on the human body, and obtaining samples such as bone tissues that can directly reflect changes in the bone microenvironment of osteoporosis patients is particularly challenging. Therefore, insufficient sample sizes have become one of the most prominent limitations in omics research. (iii) Imbalance of study participants: Most current proteomic investigations are restricted to women. Osteoporosis is prevalent in postmenopausal women; however it is not exclusive to these women. Studies indicate that approximately 25 % of males aged 50 and above experience osteoporosis [100]. (iv) Poor clinical translation rate: A significant number of biomarkers associated with osteoporosis have been identified using omics techniques, and some of these proteins have been further investigated in subsequent cellular and animal experiments. However, most of these biomarkers remain inadequately explored and are yet to be utilized in clinical settings. (v) Limitations of single-omics: The occurrence and changes in diseases cannot be completely elucidated by single proteomics. It is challenging to gain a comprehensive understanding of the intricacies of biological systems by relying only on single-omics approach as opposed to multiple-omics [101]. (vi) Establishment and extraction of information databases: Although some progress has been made in osteoporosis proteomics, the field remains in its infancy. The establishment of a knowledge base for human genome sequencing provides a framework for genomic approaches to reveal disease processes. Obtaining useful information from complex multidimensional datasets, analyzing and annotating large-scale data generated by multi-omics methods, including proteomics, and translating them into useful information for clinical decision-making will be challenges for future research.

Given the aforementioned challenges, the following suggestions are proposed: First, it is imperative to continue the advancements in omics technology to enhance the extent and sensitivity of proteomic approaches. Second, when conducting screening and validation of biomarkers using clinical samples, on the one hand, it is crucial to determine an appropriate sample size considering factors such as the type of sample, characteristics of the omics technology, and statistical analysis methodologies. On the other hand, strict inclusion and exclusion criteria can balance the statistical data of samples, making it easier to obtain samples while minimizing the systematic errors and false-positive outcomes that may arise from high-throughput sequencing. Third, although our primary focus was women, it is important to explore doing clinical studies that include a significant number of older males with osteoporosis. Fourth, to establish a more standardized and rigorous sample selection, a more rigorous screening process, comprehensive validation analysis, an in-depth research into molecular functions, mechanisms of action, and clinical translational research are essential. Particular attention has been paid to the clinical translation of biomarkers. Fifth, by merging proteomics with other omics disciplines, a thorough understanding of an organism can be achieved through multi-omics analysis. This method enables more accurate and rapid detection and identification of markers, resulting in the early diagnosis of diseases, and providing valuable information for targeted medication and lifestyle therapies. Sixth, the ongoing advancement of artificial intelligence and machine learning technology allows the integration of proteomic with these technologies, resulting in a solid foundation for the creation and analysis of proteome information databases [102].

By discovering and overcoming existing challenges, proteomics in the field of osteoporosis treatment is expected to reach its full potential for more accurate and earlier discovery and identification of biomarkers. This will facilitate diagnosis at an early stage of disease development and guide targeted drug therapy and lifestyle interventions. This improves the quality of life of osteoporosis patients by providing individualized and precise treatment plans.

6. Summary

In summary, the rapid development of human-based proteomics in

the field of osteoporosis has significantly advanced our understanding and treatment of this global health problem. The discovery of biomarkers for osteoporosis through proteomics technology is of great clinical significance, as it can not only provide risk assessment, early diagnosis and monitoring of disease progression, but also improve the study of osteoporosis-related mechanisms. Human proteomics provides a unique perspective of bone health and disease at the molecular level, which is expected to advance our understanding of the bone microenvironment and provide new clues for the optimization of therapeutic strategies. Although some challenges remain in this field, such as the improvement of proteomic technology and the limitations of sample sources and numbers ongoing research and development endeavors will further enhance the use of human proteomics in the treatment of osteoporosis.

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The authors declare that they have no competing interests.

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