SYSTEMATIC REVIEW

Toxic Effects of *p-Cresyl sulfate* and Indoxyl Sulfate on Bone: A Systematic Review

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ABSTRACT

Introduction: Chronic kidney disease (CKD) causes the accumulation of uremic toxins such as indoxyl sulfate (IS) and *p-cresyl* sulfate (pCS), leading to bone mineral disorders due to a dysfunction in the equilibrium between bone formation and resorption. Herein, we aimed to review and compile recent experimental and clinical studies that demonstrated the effect of *p*CS and IS on bone at the system, cellular, and molecular levels.**Materials and methods:** Following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria, a systematic review was performed using three electronic databases to appraise literature published between January 2005 to June 2023 on the effects of IS and *p*CS on bone. **Results:** Twenty-two relevant articles were included: 11 in vitro, 5 animal studies and 6 patient-based. IS and *p*CS induce toxic effects in bone cells by influencing cell viability, differentiation, proliferation, oxidative stress, and cell death, leading to bone morphometry alterations and low bone turnover. Higher doses of IS are needed to induce bone toxic effects compared to pCS. IS and *p*CS affect bone cells by upregulating sclerostin and decreasing levels of DMP-1, both vital for bone mineralization. Therapeutic interventions are available to reverse the toxic effects of IS and *p*CS on bone, namely probenecid, pravastatin, resveratrol and AST-120. IS and *p*CS also potentially serve as biomarkers for CKD-related bone diseases. **Conclusion:** The available evidence shows IS and *p*CS induce toxic effects on bone through various mechanisms. Further in-depth mechanistic studies are warranted to elucidate their underlying mechanisms in inducing bone changes.
Malaysian Journal of Medicine and Health Sciences (2025) 21(4): 302-313. doi:10.47836/mjmhs.21.4.37

Keywords: Indoxyl sulfate, *p-cresyl sulfate*, Osteoblast, Osteoclast, Osteocyte

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INTRODUCTION

The kidneys are integral in maintaining healthy bone mass and structure through mechanisms that maintain calcium and phosphorus levels in the blood as well as convert vitamin D into calcitriol, which is essential for calcium absorption and skeletal integrity. When kidney function is impaired, the risk of developing bone metabolic disorders (BMD) increases (1). Decreased kidney function leads to the accumulation of protein-bound uremic toxins in the body, further impairing kidney function and affecting its ability in maintaining bone homeostasis that ultimately leads to a higher mortality rate (2).

The bone is a highly dynamic tissue with mechanical and metabolic functions that are regulated by bone remodelling through bone-forming osteoblasts, bone-resorbing osteoclasts, osteocytes, and precursors cells, including monocyte/macrophage cells and mesenchymal stem cells (MSCs). In healthy individuals, bone formation and resorption are in equilibrium, maintaining bone strength and structure to meet the body's needs (3). In BMDs, such as osteoporosis and osteomalacia, the equilibrium between formation and absorption is disturbed, leading to pathological changes in bone mass that disrupt the mechanical functionality of bones and causing the accumulation of uremic toxins (4).

Uremic toxins are metabolites of organic compounds that are eliminated via the kidneys. Uremic compounds are subdivided into small water-soluble, medium, and protein-bound compounds (5). Two protein-bound uremic toxins, p-cresyl sulfate (*p*CS) and indoxyl sulfate

(IS), have clinical importance as they accumulate in blood when kidney function declines. These compounds are derived from tryptophan and tyrosine, which are ingested through diet and are metabolised into p-cresol and indole in the gut. In a healthy person, both compounds are transported to the liver, where they are chemically modified into *p*CS and IS, before being bound to albumin for excretion via the kidneys (Fig. 1). However, when kidney function is impaired, such as in chronic kidney disease (CKD), these toxins accumulate in various organs, such as the kidneys and heart, causing irreversible functional impairment (5).

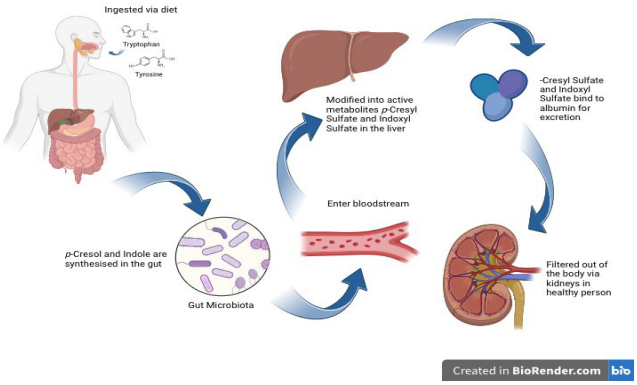

Fig. 1: The process of pCS and IS formation in the human body. Liver-modified pCS and IS are excreted by the body through the kidneys after binding with albumin.

Over 100 uremic toxins have been identified, with approximately 25% being protein-bound; these include IS and *p*CS, which affect skeletal remodelling by interfering with osteoblast and osteoclast functions in CKD (2). Despite many studies on the effects of IS and *p*CS on vital organs, only few have elucidated their effects on the bone at cellular and molecular levels. Therefore, we aimed to compile the recent findings regarding experimental and clinical studies that demonstrated the effect of *p*CS and IS on bone at the system, cellular, and molecular levels

MATERIALS AND METHODS

Search Strategy

An advanced literature search was performed in PubMed, Cochrane, and SCOPUS for studies describing the toxic effects of *p*CS and IS on bone. Keywords used were the following: 'p-Cresyl Sulfate', 'p-Cresol Sulfate', 'p-Cresyl Sulphate', 'Indoxyl Sulfate', 'Indoxyl Sulphate', 'Bone', 'Osteoblast', 'Osteoclast', and 'Osteocyte'. Selected studies were published from the 1st of January 2005 to the 31st of June 2023.

Study Selection Criteria

Two reviewers independently selected original studies using predefined eligibility criteria. Studies examined the effects of *p*CS and IS on bone metabolism through their impact on cells, such as osteoblasts, osteoclasts, osteocytes, and MSCs, and their effects on bone in animal and human studies. Duplicate review papers, poster abstracts, and papers not written in English were excluded. Reviewers agreed on which articles to include; if there was disagreement, a third reviewer was consulted to reach a consensus. This review was conducted according to the guidelines of the 2020 PRISMA statement, and their criteria for selecting papers were used to determine which articles were to be included in this review (6). Fig. 2 provides a visual summary of the number of studies included and excluded during the selection process.

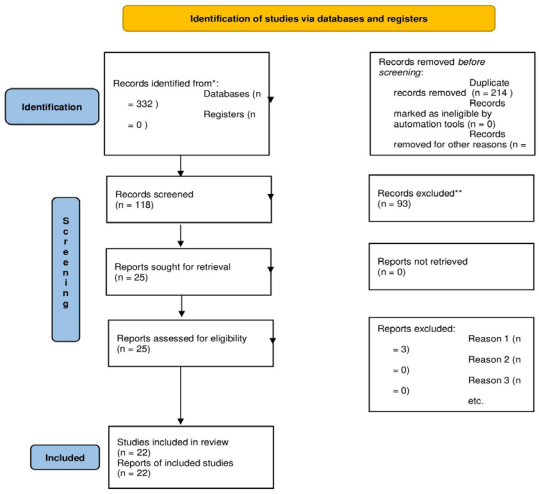

Fig. 2: PRISMA flow diagram for systematic reviews (6).

Data Extraction

To summarise the pathophysiological effects of *p*CS and IS on bone, studies were classified according to the toxins investigated, their effects *in vitro* and *in vivo*, and their key effects on bone formation and resorption. Effects were defined as cell viability, proliferation, differentiation, cellular oxidative stress, cell death, bone formation, and resorption. For *in vivo* and patient studies, effects were defined as levels of IS and *p*CS in study groups and their effects on various bone markers and morphometry.

RESULTS

The number of studies related to IS and *p*CS that can be accessed has increased, suggesting a greater worldwide recognition of both renal toxins. Of the twenty-two studies, nine were conducted in Japan, four in Taiwan, three in Korea, two in France, and one each in Brazil, China, Italy, and Thailand.

Of the twenty-two articles, eleven were conducted *in vitro*; five were on mouse osteoblasts (7-12), two on mouse monocyte/macrophages (13, 14), two on bone marrow-derived MSCs (15,16), and one on human embryonic kidney 293 (HEK293) cells (17). We included the study on HEK293 cells as it provided information on the transport of *p*CS in osteoblasts. Meanwhile, five animal studies were included; four on rat models (18-21), and one on mice (22). Overall, six

studies were patient-based; three on patients with CKD on haemodialysis (HD) (23-25), one on patients with CKD on peritoneal dialysis (PD) (26), one on patients with CKD (27), and one on patients with CKD pre-HD (28). One study combined an *in vitro* study on mouse osteocytes and an *in vivo* study on patients with CKD on PD (26). A summary of the studies' characteristics is shown in Table I.

Table I: A brief summary of each paper included in this systematic review.

Authors, Year, Reference and Type of Study	Cell Types	Objective of Study	Toxin(s)	Main Findings
Kim et al, 2013 (7) **Preclinical study**	MC3T3-E1 Mouse Osteoblast	To investigate whether IS acts as a bone toxin which induces apoptosis and inhibits differentiation in a cultured osteoblast cell line	IS	IS inhibits cell proliferation, increases oxidative stress and stimulates caspase-dependent apoptosis. IS inhibits markers of differentiation (ALP, Collagen 1, Osteonectin).
Nii-kono, 2007 (8) **Preclinical study**	Primary mouse osteoblast isolated from neonatal mouse calvariae	To examine whether IS is associated with skeletal resistance to PTH by studying the effects of IS using cultured osteoblastic cells	IS	IS significantly suppressed PTH-stimulated cAMP production, inhibited PTHR mRNA levels, increased expression of OAT3, increased cellular oxidative stress and inhibited cell proliferation.
Iwasaki, 2011 (9) **Preclinical study**	Primary mouse osteoblast isolated from neonatal mouse calvariae	To examine whether pravastatin ameliorates osteoblast dysfunction induced by uremic toxins.	IS	Pravastatin ameliorates IS-induced osteoblast dysfunction (increased intracellular oxidative stress and inhibition of cell proliferation).
Tanaka, 2013 (10) **Preclinical study**	Primary mouse osteoblast isolated from neonatal mouse calvariae	To elucidate the role of *p*CS on bone osteoblast metabolism (PTH production, ROS, apoptosis, cell viability, cell proliferation, mechanism of cell cytotoxicity) in CKD.	*p*CS	*p*CS inhibit PTH-stimulated cAMP production and gene expression in primary osteoblastic cells, induce intracellular ROS production at high levels (0.25mM), decreases cell viability of primary osteoblasts and dose-dependently promotes apoptosis, decreases osteoblastic cell proliferation, and modulates activation of JNK and p38 mitogen-activated protein kinase cascades at low levels (0.125mM).
Watanabe, 2017 (11) **Preclinical study**	Primary mouse osteoblast isolated from new born mouse calvariae	To examine the effects of IS on bone formation and bone resorption in cultures	IS	IS suppressed bone formation related genes (osterix, osteocalcin and BMP2 mRNA) IS inhibits bone resorption (IL-1) IS suppressed differentiation of macrophages into mature osteoclasts via inhibition of sRANKL expression. IS induce low bone turnover by inhibiting bone reformation and resorption in osteoclasts and osteoblast precursors
Liu, 2020 (12) **Preclinical study**	osteoblasts	To inspect the in vivo effects of an IS-induced pathological bony architecture scenario and study the molecular mechanism of osteoblast differentiation and maturation through the AhR/MAPK/Runx2 signaling pathway in vitro and to explore the possible Resveratrol (RSV) benefits on bone quality and constitution under exposure to IS.	IS	IS inhibits osteoblast differentiation (ERK1/2 and p38MAPK pathways). AhR rescues downstream expression of ERK, p38MAPK and RUNx2 induced by IS RSV attenuates effects of renal function insufficiency on bone formation in vivo and ameliorates impaired osteoblast differentiation and mineralization induced by IS.
Mozar, 2012 (13) **Preclinical study**	RAW 264.7 mouse monocyte/macrophage cell	to assess the effects of uraemic concentrations of IS on osteoclasts differentiation and bone-resorbing activity	IS	IS inhibits osteoclast differentiation (RANKL induced differentiation of macrophage cells into osteoclasts, RANKL induced differentiation of monocyte/macrophage into osteoclasts). IS reduces bone-resorbing activity of osteoclasts (inhibits activation of RANKL protein kinases, and inhibits RANKL induced DNA-binding activities of NF-kB and AP-1). IS exerts its effects after OAT mediated entry into osteoclasts.

CONTINUE

Table I: A brief summary of each paper included in this systematic review. (CONT.)

Authors, Year, Reference and Type of Study	Cell Types	Objective of Study	Toxin(s)	Main Findings
Liu, 2020 (14) **Preclinical study**	RAW 264.7 mouse monocyte/macrophage cell	To determine how IS regulates osteoclastogenesis via AhR signalling and the mechanisms affecting NFATc1 (osteoclastogenesis promoting factor) expression.	IS	IS at doses of 20 uM stimulated, but higher concentrations, inhibited osteoclast differentiation, activates AhR transcription factor signaling in osteoclastogenesis and NFATc1 are affected by IS through AhR signalling in both dose-dependent and time-dependent manner. Osteoclast differentiation increased with short-term, low dose IS exposure and decreased with long-term, high dose IS exposure.
Lanza, 2015 (15) **Preclinical study**	Bone marrow-derived mesenchymal stem cell (hMSC)	To investigate the effects of serum from uremic patients on hemodialysis on the osteogenic differentiation of hMSCs, through the analysis of various markers	IS, *p*CS	Serum from uremic patients induces, a modification of several key regulators of bone remodeling. Several bone biomarkers (osteopontin, osteocalcin, collagen type 1) are increased in cell medium, while some were decreased (BMP-2, ARS) were decreased, pointing to a reduction of bone formation favouring resorption.
Kamprom, 2021 (16) **Preclinical study**	Bone marrow-derived mesenchymal stem cell (hMSC)	To determine osteogenic differentiation ability of MSCs in the presence of IS and *p*CS.	IS, *p*CS	*p*CS reduced cell viability of MSCs , impaired osteogenic differentiation potential of MSC's, induced MSC senescence and upregulated senescence assiociataed gene (p21) during osteogenic differentiation. IS reduced cell viability of MSCs, influences osteogenic differentiation potential of MSCs, and induced MSC senescence during osteogenic differentiation.
Watanabe, 2014 (17) **Preclinical study**	Human embryonic kidney 293 (HEK293) cells	To examine the interaction or transport of *p*CS via hOAT1 and hOAT3 using HEK293 cells.	*p*CS	*p*CS is a substrate for hOAT1 and hOAT3 which is present in osteoblast cells.
Hirata, 2015 (18) **Preclinical study**	Rat model that underwent sham-operated and parathyroidectomy (PTx)	To examine whether IS exacerbates low bone turnover	IS	PTx rats with low levels of PTH develop low bone turnover and is exacerbated in the presence of IS suggesting that IS exacerbates low bone turnover by inhibiting bone formation by mechanisms unrelated to skeletal resistance to PTH.
Iwasaki, 2013 (19) **Preclinical study**	Rat model that underwent TPTx and two-stage Nx	Examine the effects of accumulated uremic toxins on bone chemical composition and elastic mechanical properties. To validate the effects of an oral uremic toxin adsorbent.	IS	low bone turnover can be seen in TPTx-Nx rats compared to TPTx rats IS is associated with increased bone fragility (increased mineral:matrix ratio, increased carbonate constitution, and decreased crystallinity) AST-120 attenuated these changes.
Meng, 2020 (20) **Preclinical study**	Calcium-deficient rats	To analyse the changed metabolites in the serum of calcium-deficient rats, to reveal calcium-deficient biomarkers, and to identify reliable biomarkers	IS	IS is elevated in serum and urine of rats with calcium deficiency. IS is a reliable biomarker to evaluate calcium-deficiency.
Iwasaki, 2006 (21) **Preclinical study**	Rat model that underwent thyroparathyrodectomy (TPTx) and two-stage nephrectomy (Nx)	To investigate the effect of IS as one of the factors responsible for bone turnover and also the effect of oral administration of AST-120 which is known to suppress the accumulation of IS	IS	Increase in IS is associated with decreased osteoblast surface per bone surface and mineral apposition ratio. Administration of AST-120 prevents accumulation of IS and suppressed the effects of above. Gene expression of PTHR, ALP and osteocalcin was reduced in TPT-Nx rats. This effect was reversed by AST-120

CONTINUE

Table I: A brief summary of each paper included in this systematic review. (CONT.)

Authors, Year, Reference and Type of Study	Cell Types	Objective of Study	Toxin(s)	Main Findings
Nam, 2018 (22) **Preclinical study**	5 months old male mice, 28 months old male mice	To determine the metabolic alterations and mechanisms that occur during age-related bone loss and to identify potential biomarkers of osteoporosis.	IS, *p*CS	Uremic toxin metabolite levels (*p*CS, IS, hippuric acid) were elevated in bone tissue,plasma and bone marrow old mice compared to in young mice. The presence of these toxins in the plasma of old mice suggests that they could be potential biomarkers for osteoporosis.
Desjardins, 2014 (23) **Prospective study**	CKD patients	To evaluate sclerostin levels in patients at different CKD stages, assess the link between sclerostin levels and biochemical parameters(IS, *p*CS) that are disturbed in renal failure and evaluate the association between sclerostin levels and mortality.	IS, *p*CS	Sclerostin levels are elevated in CKD patients (especially those on HD) and are positively correlated with IS and *p*CS levels. Elevated sclerostin levels are associated with mortality.
Chang, 2021 (24) **Cross sectional study**	HD patients	To evaluate the joint effect of *p*CS and Non-Hepatic Alkaline Phosphatase (NHALP) on Bone Fracture (BF) events in patients with HD.	*p*CS	The group of patients with high *p*CS level have a higher BF event. Higher circulating levels of *p*CS and NHALP are intrinsically associated with incremental risk of BF events.
Goto, 2010 (25) **Cross sectional study**	HD patients	To examine the relationship between IS and biochemical markers of bone turnover in HD patients to determine whether IS is involved in skeletal resistance to PTH in humans.	IS	IS may induce low bone turnover in HD patients. IS correlated negatively with bone formation markers (ALP, BAP, TRACP 5b) independent of PTH.
Yoon, 2016 (26) **Cross sectional study**	PD patients, mouse osteocytes	To determine whether DMP1 levels are associated with vascular calcification in PD patients.	IS	DMP1 levels are independently associated with the presence of vascular calcification. (do not include in results) In IS-stimulated osteocytes, mRNA and protein expression levels of DMP1 are significantly decreased compared with control osteocytes. (in bone formation)
Lin, 2014 (27) **Cross sectional study**	CKD patients	To elucidate whether intact FGF23 is correlated with selected protein-bound uremic toxins, IS and *p*CS and other independent variables in subjects with various degrees of CKD.	IS, *p*CS	Both IS and *p*CS increase within CKD patients with high levels of FGF23. Only serum IS, but not *p*CS retained an independent correlation with FGF23 in predialysis patients indicating that IS may regulate FGF23 levels independently.
Barreto, 2014 (28) **Cross sectional study**	Pre-HD CKD patients	To evaluate the association between circulating IS, biochemical parameters related to mineral metabolism and histomorphometric parameters in a cohort of prevalent, treatment-naïve pre-dialysis CKD patients.	IS	Patients at CKD stages 4 and 5 have higher levels of IS compared to stages 2 and 3. Patients at CKD stages 4 and 5 presented with higher osteoid volume, osteoblast surface, osteoclast surface, fibrosis volume and bone formation rate compared to stage 2 and 3 CKD patients.

Mechanism of transport of IS and *p*CS into bone cells

Human organic anion transporters (hOATs) comprise a group of 10 transmembrane proteins that transport IS and *p*CS across multiple tissues such as the kidneys, liver, and bone. Uremic toxins, including *p*CS and IS, compete with other molecules for the same transport pathway (29). According to five studies included in our review, hOATs is the primary mechanism through which IS and *p*CS enter osteoblasts, osteoclasts, and pre-osteoblast cells. One study reported that *p*CS uptake is significantly increased in HEK293, wherein hOAT1 and hOAT3 expression are stable. This suggests that *p*CS is a substrate for hOAT1 and hOAT3, both of which are expressed in osteoblasts (17).

Similarly, a study reported that *p*CS entered osteoblasts via hOAT (10). One study revealed that hOATs can transport IS into pre-osteoblasts using MC3T3-E1 cells (7). Two animal studies that used rat tibia samples of thyroparathyroidectomy and nephrectomy (TPT-Nx) rat models to obtain osteoblasts showed that only hOAT3 was expressed in IS toxicity (8, 21). In another study, hOAT significantly prevented osteoclast differentiation through IS (13). Probenecid, which is commonly used for gout, also acts as an OAT inhibitor, reversing the effects of IS on osteocytes (7, 10, 13); this suggests the important roles of hOATs in transporting uremic toxins across osteoblasts, osteoclasts, and tissues. hOATs play a significant role in bone cells, transporting IS and *p*CS

into osteoblasts via hOAT1 and hOAT1/3, respectively, with their entry into cells influencing cell function.

Cytotoxic effects of IS and *p*CS *in vitro*

Effects of IS and *p*CS on cell viability

This review incorporates findings from five studies examining the impact of IS and *p*CS on bone cells. Both IS and *p*CS affect osteoblasts and MSC cell viability across these studies (8, 10, 12, 14, 16). At low doses (0–1 mM) of IS, osteoblast and osteoclast viability were relatively unaffected (12, 14). However, another independent study observed decreased viability at 0.5–2 mM of IS (8). For *p*CS, a study demonstrated reduced osteoblast viability at ≥0.125 mM (10).

Another study using various IS and *p*CS concentrations on MSCs noted significant dose-dependent reductions in cell viability at 0.85 mM of *p*CS and 0.94 mM of IS (16). Additionally, Tanaka *et al.* revealed that *p*CS concentrations of 0–0.5 mM activate the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) pathway, correlating with reduced osteoblastic cell viability (10). Therefore, compared to *p*CS, higher doses of IS are required to decrease osteoblast and MSC cell viability.

Effects of IS and *p*CS on cell differentiation

Studies showed that IS and *p*CS inhibit the differentiation of precursor osteoblasts into osteoblasts. However, only IS has been shown to inhibit monocyte/macrophage differentiation into osteoclasts. Three studies were conducted on the effects of IS on precursor cells; two reported that IS suppressed soluble receptor activator of nuclear factor-kappaB Ligand (sRANKL), resulting in inhibition of osteoclast differentiation (11, 13). One study noted that compared to 0.002 mM of IS, 0.2 mM and 0.9 mM of IS inhibited RANKL-induced differentiation of monocyte/macrophages into osteoclasts (13). Another study noted that 0–0.3 mM of IS dose-dependently suppressed RANKL-induced osteoclast differentiation (11). One study also found that ≥1 mM of IS suppressed osteoblast differentiation as manifested by a significant decrease in alkaline phosphatase (ALP) levels, which reduced the expression of collagen 1 (COL1A) and osteonectin (ON) mRNA synthesis (7).

Liu *et al.* conducted two studies on osteoblast and osteoclast differentiation using IS and noted that doses ≥0.5 mM inhibited phosphorylation of extracellular signal regulated kinase 1/2 (ERK 1/2) and p38 MAPK pathways through the aryl hydrocarbon receptor (AhR) pathway, thereby inhibiting osteoblast differentiation. Exposure to <0.1 mM of IS for 3 days increased the expression of nuclear factor of activated T-cells 1 (NFATc1) and osteoclast differentiation, while exposure to higher doses (>0.5 mM) for 5 days induced NFATc1 degradation and suppressed osteoclast differentiation through AhR activation (12, 14).

Studies on the effects of IS and *p*CS on MSCs showed that both toxins inhibited osteogenic differentiation as manifested by increased and reduced levels of several bone markers. Kamprom *et al.* demonstrated that IS induced the reduction of COL1A1 expression, while the expression of other osteogenic-associated genes, such as runt-related transcription factor 2 (RUNX2) and osteopontin, remained unchanged at day 7 and 14 of differentiation in MSCs at doses of 0.47–0.94 mM. IS reduced ALP expression at day 7 and 10 of MSC differentiation, while 0.12 mM of IS induced low alizarin red staining (ARS). Furthermore, IS showed negative ARS during MSC differentiation, implying that IS inhibits MSC-osteoblastic differentiation (16). Lanza *et al.* also observed a decrease in ARS and bone morphometric protein-2 (BMP-2) levels when exposed to uremic serum, which was obtained from patients with uraemia undergoing HD, combined with 0.08 mM of IS and *p*CS during osteogenic differentiation. Thus, it is difficult to ascertain whether either toxin induced these effects (15). Taken together, although IS inhibition of osteoclast and osteoblast differentiation was mediated by the AhR receptor, signalling pathways and bone protein expression are affected depending on the cell type.

Effects of IS and *p*CS on cell proliferation

Both IS and *p*CS exert inhibitory effects on cell proliferation within osteoblasts. Among the included studies, three reported that IS affected osteoblast proliferation, while one reported that *p*CS inhibited osteoblast proliferation (7, 9, 18). *p*CS induced a dose-dependent attenuation of osteoblast proliferation, which was notably seen at doses >0.125 mM (10). Notably, the drug pravastatin, which is a lipid-lowering drug, emerged as a potential countermeasure to osteoblast proliferative repercussions induced by IS (9). Similar to the effects of IS and *p*CS on cell viability, higher doses of IS are needed to decrease cell proliferation compared to that of *p*CS.

Effects of IS and *p*CS on cellular oxidative stress

Reactive oxygen species (ROS) are highly reactive chemicals formed from a diatomic oxygen and include superoxide radicals, hydrogen peroxide, hydroxyl radicals, singlet oxygen, peroxyl radical, alkoxyl radical, lipid hydroperoxide, peroxynitrite, hypochlorous acid, and ozone. ROS activates osteoclast differentiation and osteocyte apoptosis while inhibiting osteoblast activity, resulting in bone resorption and inhibition of bone formation. Elevated levels of these metabolic by-products cause harm to important cellular structures such as proteins, lipids, and nucleic acids (30, 31).

IS and *p*CS both induce oxidative stress in osteoblasts. Three studies reported that IS induced a concentration-dependent elevation of intracellular ROS levels in osteoblasts (7, 8, 21). One study observed that *p*CS increased intracellular ROS levels in osteoblast cultures

at concentrations of ≥0.25 mM but not at lower doses (10). Meanwhile, three preclinical studies found that oxidative stress was reduced by adding OAT inhibitors such as probenecid and pravastatin (7, 9, 10). Other compounds that reduce ROS levels include antioxidants and transporter inhibitors that inhibit cellular cyclic adenosine monophosphate (cAMP), which prevents the generation of free radicals (8). Therefore, ROS levels in osteoblasts are increased in the presence of IS and *p*CS due to increased cellular oxidative stress.

Effects of IS and *p*CS on apoptotic cell death

Apoptosis is programmed cell death due to the regulated activation of a pre-existing death programme encoded in the genome (32). IS and *p*CS induce osteoblastic apoptotic cell death through various pathways. IS increases levels of pro-apoptotic markers (caspase-3/7, BAX, and p53) and decreases anti-apoptotic marker (BCL2) levels, suggesting the upregulation of apoptotic cell death in osteoblasts (7). Using DNA fragmentation as a hallmark of apoptosis, Tanaka *et al.* discovered that ≥0.125 mM of *p*CS caused a dose-dependent acceleration of DNA fragmentation. Additionally, the same study found that only doses of IS ≥0.5 mM resulted in a significant upregulation of DNA fragmentation, whereas lower doses did not produce a similar effect (10). Therefore, IS and *p*CS induce osteoblastic apoptotic cell death through distinct pathways that involve pro-apoptotic marker modulation and DNA fragmentation.

Effects of IS and *p*CS on bone formation and resorption markers

***In vitro* study**

Four preclinical studies reported that IS and *p*CS induce toxic effects on various bone markers involved in bone formation *in vitro*, leading to low bone turnover. One study demonstrated that the combination of IS or *p*CS with parathyroid hormone (PTH) in MSCs markedly reduced levels of sRANKL, which decreased the sRANKL/osteoprotegerin ratio and improved bone formation on the 3rd day of differentiation. However, on the 10th day of differentiation, levels of BMP-2 and ARS decreased, indicating reduced bone formation and favouring resorption (15). Tanaka *et al.* noted that ≥0.125 mM of *p*CS inhibited PTH-induced intracellular cAMP production and PTH receptor (PTHR) mRNA (10). In comparison, Nii-Kono *et al.* discovered that 0.05 mM of IS only increased ROS levels but not PTH-stimulated cAMP production; however, at 2 mM, IS significantly suppressed PTH-stimulated intracellular cAMP production (8). This demonstrates that compared to IS, lower doses of *p*CS are required to induce changes in cAMP production. One study noted decreased levels of bone formation markers (osterix, osteocalcin, and BMP2 mRNA) when 0.03–0.3 mM of IS was added to primary mouse osteoblasts (11). Therefore, IS and *p*CS decreases levels of bone markers associated with bone formation through different cellular pathways.

***In vivo* study**

Iwasaki *et al.* conducted two animal studies using TPTx-Nx rat models to mimic hypoparathyroidism and CKD, respectively, in humans. In that study, serum levels of uremic toxins, including IS, increased following downregulation of gene expression of proteins associated with bone formation such as osteocalcin, PTHR, and ALP. These findings suggest that IS downregulates bone formation and is associated with low bone turnover. When kremezin (AST-120), which is an oral adsorbent used to improve CKD symptoms, was administered in TPT-Nx rats, serum levels of IS decreased, and reversal of gene expression occurred (19, 21). Another animal study on IS use on rats after parathyroidectomy (PTx) showed that serum levels of bone formation markers (PTH, calcium, and ALP) were significantly decreased in IS-stimulated PTx-treated rats. This shows that increasing IS dosage decreases levels of bone formation markers, suggesting inhibition of bone formation (18). Therefore, increased levels of IS are associated with suppressed bone formation and low bone turnover as demonstrated through the downregulation of gene expression and reduction of levels of bone formation markers. There were no studies conducted using *p*CS *in vivo*; therefore there was no information regarding the effects of *p*CS on bone formation and bone markers.

Patient Study

Two studies on bone formation had different findings. One cross-sectional study comprised of 47 patients on HD reported that IS is negatively correlated with ALP and bone alkaline phosphatase levels and independent of PTH-induced skeletal resistance. However, the same study found no correlation between levels of IS and tartrate resistant acid phosphatase 5B, which is a bone resorption marker. This suggests that increased IS levels induce low bone turnover in patients on HD (25).

Another cross-sectional study that included 49 patients with CKD stages 2 to 5 noted patients with CKD stages 4 and 5 had higher levels of IS than those with CKD stages 2 and 3. Interestingly, patients with CKD stages 4 and 5 also had higher values for osteoid volumes, osteoblast surfaces, osteoclast surfaces, fibrosis, and bone formation rate than those with CKD stages 2 and 3. This is likely due to other biochemical parameters related to bone mineral metabolism, such as PTH and vitamin D-insufficiency, and increased synthesis of Wnt pathway inhibitors. Therefore, they concluded that higher levels of IS were associated with a higher bone formation rate possibly due to increased skeletal resistance to PTH or via vitamin D inhibition (28). However, as the number of participants (n = 49 and 47) for both studies was small, the results should be interpreted with caution as the sample size is not representative to the population being studied.

The relationship between bone formation and IS remains unclear, with one study reporting IS causes low bone

turnover in patients on HD, while another suggesting a complex interplay between IS accumulation and various bone metabolic factors that leads to altered bone formation rates in pre-HD patients across different CKD stages.

In vivo studies on bone formation and resorption show that IS and *p*CS suppress bone formation and induce low bone turnover. In patient studies, there are likely other parameters that influence bone formation, including PTH and vitamin D, that improve bone formation, thereby increasing bone turnover in late-stage CKD.

Levels of IS and *p*CS as potential biomarkers in CKD rat models and patients with CKD

One preclinical study noted that IS and *p*CS levels were higher in bone tissues, plasma, and bone marrow of 28-month-old mice than in 5-month-old mice. As primary osteoporosis is age-related bone loss, the results suggest that IS and *p*CS are potential biomarkers for osteoporosis (22). Long-term calcium deficiency is associated with decreased bone mineral density, such as in osteoporosis. Meng *et al.* found that rats with calcium deficiency had increased IS levels in serum and urine which can serve as a potential biomarker for calcium deficiency, as assessing levels of IS in urine rather than in serum is non-invasive (20). In patient studies , three cross-sectional studies observed that IS and *p*CS levels were elevated in CKD along with decreased levels of bone markers and increased oxidative stress, inflammation, and incidence of bone fractures (24, 27, 28). Thus, these studies suggest that IS and *p*CS can be potential biomarkers for calcium deficiency and CKD-related osteoporosis.

Relationship of IS and *p*CS with other bone markers

As levels of IS and *p*CS are increased in patients with CKD, levels of other biomarkers, such as fibroblast growth factor 23 (FGF23) and sclerostin, are also increased. One cross-sectional study noted that IS and *p*CS levels are elevated in patients with CKD with elevated levels of FGF23. FGF23 is a phosphaturic factor secreted by osteoblasts that inhibits phosphate reabsorption, causing urinary excretion of phosphate. Additionally, FGF23 is a central regulator of mineral metabolism, and its levels gradually increase with declining renal function (27). A prospective study discovered that IS and *p*CS levels are positively correlated with elevated levels of sclerostin, which is produced by osteocytes and acts as a soluble inhibitor of osteoblast function, in patients with CKD, and that increased levels sclerostin also correlates with increased FGF23, and IL-6 levels (23). One cross-sectional study observed the relationship of dentin matrix protein 1 (DMP1), which plays an essential role in bone and dental mineralisation, in osteocytes of patients with PD with IS and found that IS significantly decreases protein and mRNA expression of DMP1 compared with control osteocytes (26). Therefore, the increase in IS and *p*CS levels in patients with CKD corresponds to increased FGF23 and sclerostin levels and decreased DMP1 levels, showing their potential to influence bone metabolism and signalling pathways.

Effects of IS on bone morphometry

Two preclinical studies demonstrated that the accumulation of IS can induce alterations in bone histomorphometry. Iwasaki *et al.* showed that elevated IS levels were associated with reduced osteoblast surface per bone surface and mineral apposition ratio, indicating a reduction in bone health (19). Additionally, another study by Iwasaki *et al.* showed that IS exerts a detrimental influence on bone mechanical properties through alterations in its chemical composition, increased mineral-to-matrix ratio, increased carbonate content, and reduced crystallinity; however, these effects may be ameliorated through AST-120 administration (21). Notably, patients with CKD exhibited bone morphometric changes similar to those observed in *in vivo* studies following exposure to IS.

DISCUSSION

Elevated levels of IS and *p*CS are caused by reduced kidney function in patients with CKD. We systematically analysed studies on uremic toxins and their impact on bone cells to determine whether and how these toxins could contribute to the development and progression of bone diseases in patients with CKD. Within the context of osteoblasts, both IS and *p*CS enter cells via hOATs (7, 8, 10, 13, 17, 21). These uremic toxins increase oxidative stress and upregulate apoptosis at increased concentrations (7-10), reducing cellular viability and proliferation (7-10, 12, 14, 16, 18). Notably, IS requires a higher concentration to induce changes in cellular proliferation compared to *p*CS (7, 9, 10, 18), although it remains unclear whether these concentrations are physiologically relevant in real-world clinical settings. Additionally, the effects of *p*CS on osteoclasts remain relatively unknown in contrast to IS, which has been studied more often. Through hOATs, IS enters osteoclasts (13), causing different outcomes in a dose-dependent manner; high doses inhibit osteoclast differentiation and resorption (11, 13, 14), while lower doses promote osteoclast differentiation (14). Regarding MSCs, both IS and *p*CS activate cellular senescence genes, resulting in a decrease in levels of bone formation markers and reducing cellular viability at high doses (15, 16).

Key signalling pathways, such as the AhR signalling and JNK and p38 MAPK cascades, regulate osteoblast and osteoclast activities (10, 12, 14). IS-mediated AhR activation attenuates ERK1/2 and p38 MAPK phosphorylation, which inhibits osteoblast differentiation (12). Low doses of IS stimulate nuclear factor of activated T-cells 1 (NFATc1) in osteoclasts, thereby increasing osteoclast differentiation. In contrast, high doses of IS inhibit NFATc1, leading to reduced osteoclast differentiation (14). *p*CS stimulates JNK and

p38 MAPK phosphorylation in osteoblasts, consequently reducing osteoblast viability (10).

Findings from both animal and patient studies are similar to the *in vitro* results, demonstrating that IS and *p*CS reduce osteoblast viability, leading to reduced osteoblast activity per bone surface, which subsequently decreases levels of bone formation markers and ultimately leads to low bone turnover; this increases bone fragility and increases the risk of bone fractures (19, 21). Low bone turnover in the early stages of CKD is influenced by the toxic effects of IS and other factors such as skeletal resistance to PTH. In contrast, higher levels of PTH in later stages of CKD cause a higher bone formation rate (33). Furthermore, the relationship between IS, *p*CS, and osteocytes are intriguing as it involves an upregulation of sclerostin, an inhibitor of osteoblast function, and a decrease in levels of DMP-1, which is essential for bone and dental mineralisation (23, 26). Anti-sclerostin and DMP-1 therapy for osteoporosis have been developed; it would be interesting to investigate their potential effectiveness in managing BMD in patients with CKD (34, 35).

Our review highlights the effectiveness of various therapeutic interventions in reversing the toxic effects of IS and *p*CS both *in vivo* and *in vitro*. Probenecid demonstrates its potential by inhibiting the transport of IS and *p*CS into osteocytes while concurrently ameliorating oxidative stress (7, 10, 13). Similarly, pravastatin exhibits similar beneficial effects to probenecid, reducing oxidative stress and reversing IS-induced inhibition on osteoblast proliferation (9). Resveratrol is another promising agent as it improves renal insufficiency and reverses IS-induced impairment of osteoblast differentiation and mineralisation (12). Furthermore, the oral adsorbent AST-120 effectively suppresses increased bone fragility caused by IS and attenuates the accumulation of IS (21).

Our review also revealed some similarities and differences across studies. As shown in Fig. 3, both IS and *p*CS were shown to inhibit bone cell viability, differentiation, and proliferation (7-16,18), generate oxidative stress leading to apoptotic cell death (7, 8, 10, 21), and decrease bone turnover (8, 10, 11, 15, 18, 19, 21, 25, 28). Notably, elevated levels of IS and *p*CS were identified in rat models and patients with CKD (20, 22-24, 27, 28), with distinct mechanisms of cellular entry through hOATs (IS via hOAT3 and *p*CS via both hOAT1 and hOAT3) (7, 8, 10, 13, 17, 21).

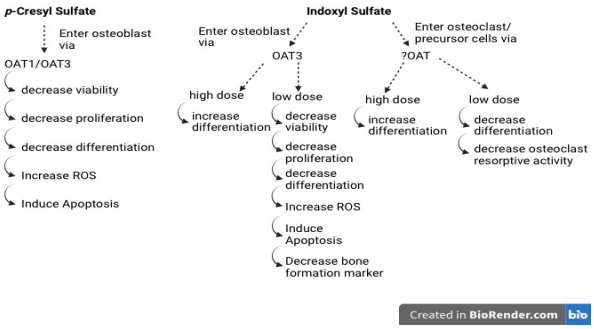

Fig. 3: Summary of the effects of pCS and IS on bone cells.

Gaps and Limitations

The authors recommended investigating how IS and *p*CS cause toxic effects on bone cells by inducing oxidative stress that lead to cell death and evaluating the long-term impact of these toxins on cellular cytotoxicity and skeletal resistance (18, 21, 23-25, 27) using human osteocytes. The authors also recommended exploring therapeutic methods to counteract the toxicity of IS and *p*CS pre-clinically and conducting clinical trials to establish them as biomarkers in skeletal events (26). However, patient studies included in this review were limited by their cross-sectional nature and small sample sizes (23-28).

Office of Health Assessment and Translation (OHAT) Risk of Bias

The Office of Health Assessment and Translation (OHAT) Risk of Bias Tools was employed in each paper included in this review (36). As shown in Table II, most studies exhibited a likely low risk of bias in the selection bias criteria due to the absence of blinding. In contrast, attrition bias was evident in some instances due to subject loss during the study, suggesting a high risk of attrition bias. In summary, the studies included in this review generally demonstrate a low or probable risk of bias.

Table II: Risk of Bias conducted for each paper included in this review according to the OHAT Risk of Bias Tools. Dark green indicates definitely low risk, light green in indicates probably low risk, pink indicates probably high risk and red indicates definitely high risk while white indicates not relevant to study (36).

Author, Year, Reference	Selection Bias	Confounding Bias	Performance Bias	Attrition Bias	Detection Bias	Selective Reporting Bias	Other Bias
Kim et al., 2013 (7)							
Nii-Kono et al., 2007 (8)							
Iwasaki et al., 2011 (9)							
Tanaka et al., 2013 (10)							
Watanabe et al., 2017 (11)							
Liu et al., 2020 (12)							
Mozar et al., 2012 (13)							
Liu et al., 2020 (14)							
Lanza et al., 2015 (15)							
Kamprom et al., 2021 (16)							
Watanabe et al., 2014 (17)							
Hirata et al., 2015 (18)							
Iwasaki et al., 2013 (19)							
Meng et al., 2020 (20)							
Iwasaki et al., 2006 (21)							
Nam et al., 2018 (22)							
Desjardins et al., 2014 (23)							
Chang et al., 2021 (24)							
Goto et al., 2010 (25)							
Yoon et al., 2016 (26)							
Lin et al., 2014 (27)							
Barreto et al., 2014 (28)							

CONCLUSION

After entering bone cells through hOATs, IS and *p*CS induce toxic effects on bone through various mechanisms such as the inhibition of cell viability, differentiation, proliferation, and the stimulation of cellular oxidative stress and apoptotic cell death, leading to alterations in bone morphometry and low bone turnover. As their levels increase with BMDs, calcium deficiency, and age, these uremic toxins can be considered as potential biomarkers for CKD-related bone diseases. Further in-depth mechanistic research conducted to elucidate the effects of IS and *p*CS *in vitro* and *in vivo* is warranted to better understand their toxic effects on bone and their underlying mechanisms in inducing bone changes.

ACKNOWLEDGEMENT

The authors acknowledge the financial support from the Ministry of Higher Education Malaysia through the Fundamental Research Grant Scheme (FRGS) with grant number FRGS/1/2021/SKK0/UNIMAS/01/2 and

Universiti Malaysia Sarawak for supporting this project.

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