Fetuin-A Regulation of Calcified Matrix Metabolism

Willi Jahnen-Dechent, Alexander Heiss, Cora Schäfer, Markus Ketteler

Abstract: The final step of biomineralization is a chemical precipitation reaction that occurs spontaneously in supersaturated or metastable salt solutions. Genetic programs direct precursor cells into a mineralization-competent state in physiological bone formation (osteogenesis) and in pathological mineralization (ectopic mineralization or calcification). Therefore, all tissues not meant to mineralize must be actively protected against chance precipitation of mineral. Fetuin-A is a liver-derived blood protein that acts as a potent inhibitor of ectopic mineralization. Monomeric fetuin-A protein binds small clusters of calcium and phosphate. This interaction results in the formation of prenucleation cluster-laden fetuin-A monomers, calciprotein monomers, and considerably larger aggregates of protein and mineral calciprotein particles. Both monomeric and aggregate forms of fetuin-A mineral accrue acidic plasma protein including albumin, thus stabilizing supersaturated and metastable mineral ion solutions as colloids. Hence, fetuin-A is a mineral carrier protein and a systemic inhibitor of pathological mineralization complementing local inhibitors that act in a cell-restricted or tissue-restricted fashion. Fetuin-A deficiency is associated with soft tissue calcification in mice and humans. (Circ Res. 2011;108:1494-1509.)

Key Words: calcification ■ mineral metabolism ■ plasma proteins

Fetuins are vertebrate plasma proteins. Fetuin-A/\(\alpha_2\)-Heremans Schmid (HS) glycoprotein homologues occur in reptiles, fish, birds, marsupials, and mammals. Bovine fetuin (derived from the Latin word fetus) was first described in 1944 by Pedersen\(^1\) as the most abundant globular plasma protein in fetal calf serum. The human species homologue was independently identified by Heremans\(^2\) and Schmidt and Bürgi.\(^3\) It was later named \(\alpha_2\)-HS-glycoprotein by Schultzze\(^4\) in honor of two of the original codiscoverers. The name also indicates that \(\alpha_2\)-HS glycoprotein comigrates with the \(\alpha_2\)-globulin fraction of serum proteins in cellulose acetate electrophoresis.

This protein is confused in the literature with \(\alpha_2\)-Z-glycoprotein/Zn \(\alpha_2\) glycoprotein (Z for zinc-binding) or...
**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>(\alpha_{2})-HS-glycoprotein</td>
<td>(\alpha_{2})-Heremans Schmid glycoprotein/fetuin-A</td>
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<tr>
<td>AB</td>
<td>Apoptotic body, membrane enclosed fragments of cells undergoing apoptosis</td>
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<tr>
<td>AFP</td>
<td>Alpha-fetoprotein</td>
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<tr>
<td>AHSG</td>
<td>Genetic symbol for (\alpha_{2})-HS-glycoprotein</td>
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<tr>
<td>ANK</td>
<td>Ankylosing spondylitis gene, mutation of this gene causes spondyloarthritis</td>
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<tr>
<td>BCP</td>
<td>Basic calcium phosphate (octacalcium phosphate or hydroxyapatite) as opposed to acidic calcium-like phosphate calcium pyrophosphate dihydrate</td>
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<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>BMP-R1</td>
<td>BMP receptor-1</td>
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<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>CPM</td>
<td>Calcifying nanoparticles</td>
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<tr>
<td>CTP</td>
<td>Calciprotein monomer</td>
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<tr>
<td>CPP</td>
<td>Calciprotein particle</td>
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<tr>
<td>CUA</td>
<td>Calcific uremic arterioleopathy/calciphylaxis</td>
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<tr>
<td>DLA</td>
<td>Dynamic light scattering</td>
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<tr>
<td>ENPP1</td>
<td>Extracellular nucleotide pyrophosphatase/phosphotransferase-1, a pyrophosphate-cleaving enzyme</td>
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<tr>
<td>EPIC</td>
<td>European Prospective Investigation into Cancer and Nutrition study involving 27,548 subjects</td>
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<td>FABP</td>
<td>Fatty acid binding protein</td>
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<td>FMC</td>
<td>Fetuin mineral complex</td>
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<td>HMGB1</td>
<td>High-mobility group protein B1, a proinflammatory nuclear protein</td>
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<tr>
<td>HRG</td>
<td>Hidde-rich glycoprotein, a fetuin-related protein</td>
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<tr>
<td>KNG</td>
<td>Kinogen, a fetuin-related protein</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide, a strong inflammatory agent from bacterial cell walls</td>
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<tr>
<td>MGP</td>
<td>Matrix GLA protein</td>
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<td>MO</td>
<td>Monocyte</td>
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<tr>
<td>MIA</td>
<td>Malnutrition-inflammation-atherosclerosis syndrome</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MP</td>
<td>Macrophage</td>
</tr>
<tr>
<td>MV</td>
<td>Matrix vesicles, mineral-laden vesicles believed to mediate bone mineralization</td>
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<tr>
<td>NCP</td>
<td>Noncollagenous proteins (from bone)</td>
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<tr>
<td>NLRP3</td>
<td>NOD-like receptor 3</td>
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<tr>
<td>NMP</td>
<td>Nucleotide monophosphate</td>
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<tr>
<td>NTP</td>
<td>Nucleotide triphosphate</td>
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<tr>
<td>OPN</td>
<td>Osteopontin</td>
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<tr>
<td>PD</td>
<td>Peritoneal dialysis</td>
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<tr>
<td>Pit-1</td>
<td>Sodium/phosphate cotransporter</td>
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<tr>
<td>PP</td>
<td>Pyrophosphate a potent crystal growth inhibitor and anticalcification agent</td>
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<tr>
<td>PS</td>
<td>Phosphatidylserine, membrane phospholipid exposed on the cell surface during apoptosis</td>
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<tr>
<td>Runx2/Cbfa-1</td>
<td>Runt-related transcription factor 2/core binding factor 1, an osteoblastic transcription factor</td>
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<tr>
<td>SANS</td>
<td>Small-angle neutron scattering, an analytical technique to determine molecular structure</td>
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<tr>
<td>SAXS</td>
<td>Small angle x-ray scattering, an analytical technique to study crystal growth at high resolution</td>
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\(\alpha\)-fetoprotein, two unrelated entities. Fetuin-A is member of a family of four structurally related plasma proteins containing cystatin-like protein domains. Cystatins can inhibit cysteine peptidases of the papain, calpain, cathepsin, and caspase families and play key roles in a wide array of physiological processes as well as in disease. The cystatin family harbors type 1 (mainly intracellular proteins), type 2 (mainly extracellular proteins), and type 3 cystatins (plasma proteins). Figure 1 shows cartoons of the type 3 family members fetuin-A/\(\alpha_{2}\)-HS glycoprotein, fetuin-B, histidine-rich glycoprotein, and kinogen. Cystatin domain 1 in fetuin-A is strongly negatively charged with a high affinity for calcium-rich minerals.5

**Fetuin-A Biosynthesis**

Fetuins are highly expressed liver-derived plasma proteins bearing posttranslational modifications proteolytic processing,6,7 complex glycosylation,8–10 phosphorylation (Ser and Thr),5,11–15 and sulfation.16 Human fetuin-A/\(\alpha_{2}\)-HS glycoprotein is processed from a single chain precursor to the mature circulating two-chain form.6 Human fetuin-A is susceptible to further proteolytic cleavage in septicemia,7 and bovine fetuin-A is processed by matrix metalloproteinases.15 Figure 2 illustrates secondary modifications and allelic variants identified in human fetuin-A/\(\alpha_{2}\)-HS glycoprotein. These modifications may regulate protein expression levels, stability, and biological activity. Phosphorylation is indispensable for fetuin-A interaction with the insulin receptor,17,18 whereas phosphorylation seems not to be required for mineral interaction.19 Desialylation will result in immediate hepatic clearing through the asialoglycoprotein receptor.20–22

**Binding Properties of Fetuin-A**

The type 3 members of the cystatin superfamily are glycoproteins produced mainly in the liver, which circulate in plasma at high concentrations. Fetal calf serum contains more fetuin-A than albumin. Apart from the vasculature, fetuins are present throughout the extracellular spaces and the extracellular matrix. Given the high expression levels and the many possible ways of molecular interaction with multiple ligands, it is fair to assume that fetuins primarily exercise carrier and scavenger functions like albumin. This poses an important complication for ex vivo experimentation, because crude fetuin preparations contain impurities. Like pure albumin preparations, pure fetuin preparations are difficult to make. High-abundance plasma protein preparations are notoriously
contaminated with lower-abundance biological molecules that copurify either because of natural association or because of mere coincidence. Various growth factors or growth-promoting substances associate with crude fetuin preparations and form the basis of the “enigmatic growth promoting properties” of fetuin in cell culture.\textsuperscript{23}

Whether associations of fetuin-A with certain ligands are physiologically relevant is a matter of controversy and is almost impossible to decide unless genetic models are developed to test such interactions in vivo. We remind readers to heed the old wisdom of Racker\textsuperscript{24} that also became part of the “10 commandments” of Kornberg,\textsuperscript{25} “Do not waste clean thinking on dirty enzymes.” Most commercial preparations of fetuin-A on the market today do not go much beyond the quality of “fetuin” from 1944,\textsuperscript{1} which is better viewed as a “protein concept” like “globulin” or “albumin” rather than a clean product. We distinguish between fetuin and fetuin-A whenever possible. Also, before the year 2000 when fetuin-B was cloned in silico\textsuperscript{26} and the year 2003 when it was finally shown to be expressed as a plasma protein,\textsuperscript{10} both fetuin-A and fetuin-B were collectively addressed as “fetuin,” and most fetuin sold today is not screened for the presence of fetuin-B. Studies of human α\textsubscript{1}-HS-glycoprotein/fetuin (α\textsubscript{1}-HS glycoprotein) will always signify fetuin-A; publications studying “fetuin” protein are probably dealing with fetuin-A as well, but it is impossible to exclude that fetuin-B was also studied because of the physicochemical similitude of both proteins.

Fetuin-A binding is prodigious and reaches from small molecules to entire organisms. Plasmodium sporozoites use fetuin-A binding to “hitch-hike” their way into liver cells,\textsuperscript{27} suggesting that fetuin-A function is indispensable and despite negative selection pressure has been maintained long enough for this docking mechanism to evolve. Soon after its discovery, fetuin has been shown to inhibit trypsin.\textsuperscript{28} Both positive and negative fetuin interactions with proteases are documented, including matrix metalloproteinase-9,\textsuperscript{29} matrix metalloproteinase-3,\textsuperscript{30} meprin metalloproteinases,\textsuperscript{31} the cysteine proteases m-calpain,\textsuperscript{32} cathepsin L in rat bone,\textsuperscript{33} and inhibition of recombinant human cathepsin V. Proteinase interactions are thought to be involved in the regulation of tumorigenesis and tumor progression.\textsuperscript{34,35}

Fetuin markedly accelerates incorporation of exogenous fatty acids into cellular triglycerides.\textsuperscript{36–39} Thus, fetuin may
share a function with fatty acid binding proteins, a family of abundantly expressed 14-kDa to 15-kDa proteins. Like fetuin, fatty acid binding proteins reversibly bind hydrophobic ligands, including saturated and unsaturated long-chain fatty acids, and other lipids with high affinity.

Because of their rich complex glycosylation pattern, fetuins serve as model substances for lectin and glycoprotein research. Lectin binding should always be seriously considered when fetuin binding to cells and to the extracellular matrix is studied. On a practical note, the strong binding of pertussis toxin to terminal sialic acid residues in fetuin form the basis of a Food and Drug Administration-approved pertussis toxin test. Fetuin-A sequestration of lectins proved a major complication in experimental cytotoxic therapy using cancer cell–specific antibodies coupled to the Ricinus communis agglutinin ricin. The immunotoxins were rapidly cleared by the asialoglycoprotein receptor and caused liver toxicity.

In a search for natural transforming growth factor-beta (TGF-β) receptor antagonists, a sequence homology was found between TGF-β receptor type II and fetuin-A. The TGF-β receptor II homology 1 domain from fetuin bound preferentially to bone morphogenetic protein (BMP)-2. Full-length fetuin-A bound directly to TGF-β1 and TGF-β2 and with greater affinity to the TGF-β-related BMP-2, BMP-4, and BMP-6. Finally, and likely impinging on fetuin’s role in mineralization biology, fetuin or neutralizing anti-TGF-β antibodies blocked osteogenesis and deposition of calcium-containing matrix in mineralizing cell cultures. An altered bone phenotype in fetuin-A–deficient mice (Ahsg$^{-/-}$) was explained accordingly in terms of failure to block TGF-β–dependent signaling in osteoblastic cells. Tumorigenesis experiments using Ahsg$^{-/-}$ mice further supported the hypothesis that fetuin-A is an antagonist of TGF-β in vivo, in that it inhibited intestinal tumor progression.

### Antiinflammatory Role of Fetuin-A
Fetuin-A, one of the most abundant fetal plasma proteins, was found to be essential for the inhibition of the proinflammatory cytokine tumor necrosis factor production by spermine and its synthetic analogues. Accordingly, the strong fetal expression of fetuin and spermine have been associated with the tolerance of the fetus, “nature’s transplant,” by mothers. The strong antiinflammatory effects of fetuin were verified in vivo using several models of inflammation, including lipopolysaccharide-induced miscarriage in rats, carrageenan injection, cerebral ischemic injury in rodents, and cecal ligation and puncture in mice. In all cases fetuin-A was associated with reduced inflammatory response and increased survival, and administering additional fetuin generally improved outcome.

Thus fetuin-A generally may be regarded as antiinflammatory. The antiinflammatory property of serum α2-HS glycoprotein/human fetuin-A was further supported by the demonstration that fetuin-A is a potent and specific crystal-bound inhibitor of neutrophil stimulation by hydroxyapatite crystals. Calcium phosphate crystals induce proinflammatory cytokine secretion through the NLRP3 inflammasome in monocytes/macrophages, cell death in human vascular smooth muscle cells, and cell activation in chondrocytes.

Antiapoptotic activity of fetuin-A has been observed in smooth muscle cells and dampering of the cell-specific responses would generally be expected to alleviate the detrimental consequences of local inflammation, cell death, and cartilage degradation. The proven protective function of fetuin-A in many animal models of inflammation, the inhibition of proinflammatory compounds, and the inhibition of crystal-induced neutrophil activation collectively suggest that fetuin-A may generally protect during pathological mineralization as well.
**Fetuin-A in Metabolic Syndrome**

Several clinical studies proposed that high-serum fetuin-A levels are associated with metabolic syndrome (MetS) and that fetuin-A therefore may present a risk factor for MetS. The association of fetuin-A, insulin signaling, diabetes type 2, and MetS dates back to a publication in 1989 stating that pp63, a liver-secreted phosphoprotein, inhibited insulin receptor and downstream substrate phosphorylation signaling. The cDNA sequence of pp63 was identified as rat fetuin-A, and it was disputed whether the activity tested in the original article was actually rat fetuin-A or a copurified protein. We tested authentic human fetuin-A and found that, if any, inhibitory activity at the insulin receptor and downstream substrate level. We analyzed phosphorytrosine modification of cellular proteins in rat fibroblasts expressing the human insulin receptor and could not detect any robust and reproducible inhibition of insulin signaling by human or bovine fetuin including phosphofetuin-A immunoaffinity purified from HepG2 cells. Testing of fetuin-A in relevant cells showed no classical insulin receptor inhibition, but showed unexplained downregulation of insulin-stimulated Elk1-phosphorylation.

In another series of experiments, commercial bovine fetuin and supernatants of baculovirus-infected insect cells expressing human fetuin-A did inhibit insulin receptor signaling. Furthermore, fetuin-A was shown by immunoprecipitation to directly interact with the insulin receptor. These researchers also showed that fetuin-A-deficient mice maintained on a mixed genetic background of C57BL/6N and 129Sv had improved insulin response, resistance to weight gain, protection against obesity, and protection against insulin resistance associated with aging. We back-crossed these mice that were originally generated in our laboratory onto pure C57BL/6N as well as DBA/2 genetic background, and we could not detect evidence of diabetes-associated symptoms in the genetically more homogeneous mice. The genetic background of mice and even substrains of mice differ with respect to insulin sensitivity. A deletion variant of nicotinamide nucleotide transhydrogenase that has spontaneously occurred in C57BL/6J, but not in C57BL/6N strains, is thought to influence insulin signaling. Therefore, sibling mice from the same colony should be ideally used when comparing the influence of single gene deletions on insulin sensitivity. Mice with homogeneous defined genetic background were not yet available at the time of the first study in the case of fetuin-A-deficient mice.

Thus, it is unclear if fetuin-A is a cause or consequence of high-caloric feeding in mice or whether the association of high-serum fetuin-A and MetS is a bystander phenomenon. First, fetuin-A is a highly expressed constitutively secreted hepatic serum glycoprotein. Using nuclear run-on assays, LeCam et al. determined that the rat fetuin-A/pp63 promoter strength was approximately three-times to four-times that of the strong liver-specific albumin promoter. Second, fetuin-A is traditionally regarded as one of the few negative acute phase proteins and strong associations between low-serum fetuin-A levels and inflammatory markers like C-reactive protein have been published. Thus, downregulation rather than upregulation of fetuin-A during “fat inflammation” in obesity-related insulin resistance would be expected. Therefore, it seems counterintuitive that fetuin-A levels would be increased in MetS. Recent work showed that fetuin-A induced inflammatory cytokines, and that one of the major mediators of inflammatory cytokine action, NFκB, further upregulated hepatic fetuin-A synthesis. The conflicting data on fetuin-A regulation clearly need to be reconciled in terms of timing of events and causal relationships vs mere association. The association of fetuin-A serum levels with MetS and type 2 diabetes in humans rests on single measurements, and most studies did not correct for a major confounder, total liver protein synthesis. Therefore, we suggest that association studies should at least be normalized to serum albumin as an indicator of total liver protein synthesis, unless serum albumin should also be called a risk factor of MetS.

**Role of Fetuin-A in Mineralization Biology**

We started working on fetuin-A as part of an ongoing research project studying the structure and function of type 3 members of the cystatin superfamily. At that time, fetuin-A/α2-HS glycoprotein was a “structure in search of a function.” Intrigued by the description of rat fetuin as a major mediator of inflammatory cytokine action, NFκB, and by the report on the identity of fetuin as a natural inhibitor of insulin receptor kinase inhibitor, we studied fetuin-A binding to cell membranes and extracellular targets. Binding to mineralized bone matrix as described by Triffitt et al. turned out to be the most robust binding phenomenon by far. The high affinity of fetuin-A for bone mineral mediates selective fetuin-A accumulation from plasma into bone. Fetuin-A in bone accounts for 25% of the noncollagenous proteins and thus is one of the two most highly abundant noncollagenous proteins. We showed that fetuin-A also has a particularly high affinity to nascent apatite mineral and is an inhibitor of de novo apatite formation from supersaturated mineral solutions. The inhibitory effect is mediated by acidic amino acids clustering in cystatin-like domain D1. This fundamental observation formed the base of two decades of fetuin-A mineralization research and has been successfully repeated by many independent laboratories. In full accord with this biochemical finding, genetic work using fetuin-A-deficient mice likewise suggested that fetuin-A is a mineral chaperone mediating the transport of mineral from the extracellular space and the general circulation.

**Fetuin-A–Mineral Complexes, Calciprotein Particles, Calcifying Nanoparticles**

Fetuin-A binds calcium phosphate and calcium carbonate with high affinity, and it binds magnesium phosphate less well. Importantly, fetuin-A only inhibits the de novo formation of calcium phosphate and does not dissolve preformed mineral. Fetuin-A is an inhibitor of mineralization in solution and of cell-mediated mineralization in that it regulates the process of matrix mineralization in rat calvaria osteoblastic cells. On addition of β-glycerophosphate and ascorbate,
Figure 3. Electron microscopic pictures of synthetic and patient-derived calciprotein particles (CPP). A, Primary calciprotein particles have a diameter of 30 nm to 150 nm and contain amorphous mineral. B, Subsequently, the primary CPP were subjected to a major structural rearrangement. The resulting secondary CPP consist of a core of crystalline basic calcium phosphate, which is covered by a layer of fetuin-A (A and B). 50 μmol/L bovine fetuin-A, 10 mmol/L CaCl₂, 6 mmol/L Na₃HPO₄, pH 7.4. These particles are stable for days at 37°C. C, Similar particles have been detected in ascites of patients with sclerosing calcifying peritonitis. Bars represent 100 nm.

Figure 4 depicts schematically the sequence of calciprotein particles formation, starting with the spontaneous formation of prenucleation clusters from metastable solutions, their sequestration to acidic aspartic acid, and glutamic acid side chains in the cystatin-like domain of fetuin-A forming mineral-laden fetuin-A monomer or calciprotein monomer. Calciprotein monomer was discovered when quantitative small-angle neutron scattering data analysis revealed that even at a fetuin-A concentration close to the stability limit of supersaturated salt solutions, only approximately one-half of the mineral ions and only 5% of the fetuin-A were contained in the calciprotein particles. The remaining supersaturated mineral ion fraction and 95% of noncalciprotein particles of fetuin-A were associated with a mineral-laden fetuin-A monomer fraction that could be separated from mature calciprotein particles by ultrafiltration through a 300-KDa cut-off membrane. Small-angle neutron scattering analysis showed that the fetuin-A monomer in this fraction was closely associated with coalesced subnanometer-size mineral ions clusters reminiscent of Ca₉(PO₄)₆PO₄ Posner clusters (Figure 4A). Fetuin-A binds to apatitic mineral surfaces, as shown in Figure 4C. Fetuin-A does not influence the formation of mineral nuclei. However, fetuin-A prevents the growth and aggregation of nuclei to larger entities and ultimately mineral precipitation. Thus, fetuin-A effectively shields spontaneously formed mineral nuclei, leading to transiently stable calciprotein particles.

A hierarchical role of fetuin-A was established in that fetuin-A was critically required during the early steps of calciprotein particles formation and stabilization and that other acidic plasma proteins including serum albumin further stabilized the initial colloids and could substitute for fetuin-A at later stages of calciprotein particles formation. These later stages were studied in detail using small-angle neutron scattering. Figure 4D illustrates the aggregation of many calciprotein monomers and other mineral nuclei into transiently stable primary calciprotein particles of initially <100 nanometers in diameter. After a lag period, these particles grow into elongated and more crystalline particles of approximately twice the initial size, termed secondary or mature calciprotein particles (Figure 4E). Thus, formation and maturation are two separate and successive processes following the principles of Ostwald ripening. Similar particles may be generated with other acidic macromolecules as well. However, fetuin-A is exceptionally potent regarding activity and specificity of inhibition. Increased fetuin-A concentration leads to smaller particles. An increased temperature, mineral ion concentration, and a reduced fetuin-A concentration,
packages VESTA,178 VMD,179 and APBS.180

respectively, all accelerate the particle ripening process, demonstrating that calciprotein particle maturation follows the Arrhenius law. Secondary calciprotein particles are stabilized by a compact outer fetuin-A monolayer against further growth (Figure 4E) for up to 30 hours at body temperature, which is ample time for clearing of calciprotein particles from circulation.

The formation and maturation of calciprotein particles can be followed by optical monitoring110 of mineralizing solutions. Supplemental Figure I (available online at http://www.circresaha.org) shows dynamic light scatter diagrams illustrating that the transformation from primary to secondary calciprotein particles is fetuin-A concentration-dependent. Higher fetuin-A concentrations cause prolonged stability of both calciprotein monomers and calciprotein particles, and thus a right-shift in the transformation curves. A clinical test could use the kinetic parameters of the precipitation reaction, thus measuring the overall calcification risk in biological fluids, eg, in the blood of dialysis patients.

Protein–mineral complexes are soluble precursors of physiological mineralization of bones and teeth as well. Recent research has shown that biomineralization starts with amorphous mineral–protein complexes containing fetuin-A.123–126 Price et al127–129 have shown that fetuin-A is critically required to direct mineralization to the interior of synthetic matrices that have size exclusion characteristics similar to those of collagen. Fetuin-A does so by selectively inhibiting mineral growth outside of these matrices. This mineralization by inhibitor exclusion is likely redundant, because fetuin-A-deficient mouse bone is mineralized perfectly well. The mice nevertheless show a bone phenotype of stunted femur length, indicating premature growth plate mineralization and disturbed osteogenic signaling.47

**Nanobacteria: A Red Herring From Mars**

Nanobacteria initially described nanoscopic life forms detected by electron microscopy in rock sediments.130 Similar entities were discovered in meteorites from Mars131 and, finally, in cell culture.132 Nanobacteria attracted a lot of scientific and economical attention as a causal agent of major diseases, including vascular calcification, kidney stones, and cancer.132–136 In the year 2000, Cisar et al137 determined that simple mixtures of phospholipids and calcium phosphate crystals closely resembled nanobacteria in that they showed life-like growth and replication. Nucleic acid sequences previously thought to be diagnostic markers of nanobacteria were in fact diagnostic of common laboratory contaminants.137 Two groups of researchers finally solved the riddle of pathological mineralizing nanobacteria.120,138 A series of experiments on the origin of putative nanobacteria showed that calcium phosphate together with proteins and further nonmineral compounds formed nanoparticles that resembled nanobacteria in shape and behavior.130 Minerals containing nanoparticles had a high binding capacity for charged molecules, including ions, carbohydrates, lipids, and nucleic acids. Depending on the exact composition, mixtures of mineral and nonmineral compounds sustained either crystallization of hydroxyapatite mineral or the formation of complex protein–mineral complexes.122,140 It was found that the main protein component of the nanobacteria was fetuin-A.120 Besides fetuin-A, serum albumin and apolipoproteins were also identified;121 hence, the term nanobacteria was exchanged for the more apt term calcifying nanoparticles, virtually identical to fetuin–mineral complexes described before.

**Calciprotein Particles Metabolism and Clearing**

Figure 5 illustrates the putative metabolism of calciprotein particles, soluble protein–mineral complexes, which are now
plasma proteins, which adhere to most materials in blood cell membranes, phagocytosis of apoptotic cells by macrophages, Fetuin-A affects microparticle phagocytosis by dendritic cells, dosed rats, in adenine-treated rats, in peritoneal dialysis patients, Fetuin-A have been detected in serum from etidronate overdose patients, the scheme is modeled after lipoprotein metabolism, a well-known transport system for other cationic, lipidic, or mineral ligands, and a few more interacting TGF-β-related cytokines mentioned earlier in this text. Given the high abundance of Fetuin-A in plasma, any clearing mechanism would have to rely on conformational or structural changes in Fetuin-A or on multivalent binding that turns low-affinity binding into high-affinity binding.

Uptake of Fetuin-A by cultured human vascular smooth muscle cells has been demonstrated, but the exact form of Fetuin-A was not determined. Clustering of Fetuin-A molecules on the surface of secondary calciprotein particles as demonstrated ideally fulfills the ligand clustering required to increase binding strength. Our preliminary results of Fetuin-A monomer and Fetuin-A containing calciprotein particle clearing in vivo show that calciprotein particles are cleared vastly more efficiently than Fetuin-A monomer. Nevertheless, specific receptors for calciprotein particle clearing discriminating against Fetuin-A monomer remain to be determined.

**Fetuin-A Knockout Phenotype**

The use of animal models has uncovered major inhibitors of pathological calcification and their mode of action. Fetuin-A is one of the major systemic inhibitors of pathological mineralization, but it is not the only one. This is vividly illustrated by the history of Fetuin-A–deficient mice (Ahsg−/−). While establishing that Fetuin-A is a regulator of mineralization, we also generated Ahsg−/− mice to test this hypothesis in an animal model. For technical reasons, the first Ahsg−/− mice had a mixed 129Sv × C57BL/6 genetic background designated 129.B6-Ahsgtm1mbl. Out of an initial colony of approximately 200 mice, only approximately 10 female ex-breeders showed a spontaneous calcification phenotype. All other mice were apparently normal. Force-feeding supra-physiological doses of active vitamin D (calcitriol) and a high-mineral diet (phosphate-rich) resulted in nephrocalcinosis and pulmonary and myocardial calcification of these mice.

Unsatisfied by this highly variable yet low-penetrating calcification phenotype, we decided to combine the Fetuin-A deficiency with the calcification-prone DBA/2 genetic background. For comparison, we also backcrossed the mice onto the widely used calcification-resistant genetic background C57BL/6 and thus generated two more Ahsg−/− mouse strains, D2-Ahsgm1wja and B6-Ahsgm1wja. The latter mice behaved like the mixed genetic background mice in that they scarcely had any spontaneous calcification. They did, however, have calcification when challenged with hemin-phrectomy and a high-mineral diet. In stark contrast, virtually all D2-Ahsgm1wja mice show spontaneous massive soft tissue calcification throughout their bodies. This strain is arguably the most calcifying mouse strain in existence in that the myocardium of D2-Ahsg−/− mice contains almost one-tenth the calcium content of normal bone. Kidney, lung, skin, brown fat, pancreas, and reproductive organs are equally strongly calcified.

![Figure 5. Hypothetical pathway of calciprotein particle (CPP) circulation and clearing by endothelial cells and tissues resident phagocytes. Fetuin-A stabilizes CPP in the circulation and mediates their efficient uptake. In healthy individuals, CPP may form spontaneously and be present in small numbers throughout all tissues, or may occur substantially where bone resorption by osteoclasts (OC) is releasing high levels of calcium and phosphate by acid-mediated mineral dissolution. CPP may be transported in the blood or retrieved locally after nearby osteoclastic resorption, and are used locally by osteoblasts (OB) during bone formation. When mineral homeostasis is severely disturbed, eg, in dialysis patients or in severely etidronate–overdosed rodents, bounts of hypercalcemia and hyperphosphatemia will result in the formation of high numbers of CPP and Fetuin-A will be consumed in the process as part of the CPP complex. CPP in the interstitial spaces will be cleared by monocytes (MC) or macrophages (MP) or by any other endocytosing cell types. Overly abundant CPP may overwhelm the clearing capacity of the reticuloendothelial system phagocytes and may result in apotosis of endothelial cells (EC), MP, and smooth muscle cells (SMC), and in deposition of calcified apoptotic cell remnants. This pathway is hypothetical and needs to be experimentally verified. It may also be of significance that many inhibitors of calcification activate monocytes/macrophages and stimulate phagocytosis; therefore, this mode of calcified remnant clearing mechanism would have to rely on conformational or structural changes in Fetuin-A or on multivalent binding that turns low-affinity binding into high-affinity binding.](http://circres.ahajournals.org/doi/fig/10.1161/CIRCRESAHA.117.312500)
Supplemental Figure II (available online at http://www.circresaha.org) shows random examples of 11-month-old wild-type or Abcc6−/− mice maintained against the genetic background C57BL/6 or DBA/2. Even from the low-resolution radiology photographs, the uniformity of genetic penetrance and the extent of the calcification in D2-Ahsg−/− mice can be readily appreciated. Kidney and skin calcification are easily visible. Details may be glanced at higher magnifications of the electronic pictures provided with this article. We point out that the pictures were taken of live anesthetized mice. Therefore, myocardium, lung, and pancreas calcification are invisible or blurred because of breathing motion of the chest region. Surprisingly, the soft tissue calcification is not lethal like the arterial calcification of matrix GLA protein–deficient mice.157 Calcification does, however, greatly compromise reproduction in mice aged 6 months and older. The 1-year survival rate of D2-Ahsg−/− mice in our specified pathogen-free colony is 60% to 70% of all born pups, compared to 90% to 95% in DBA/2 wild-type mice.

These results showed that fetuin-A is a systemic and soluble inhibitor of pathological mineralization that is backed-up by other genetic factors rendering mouse strains prone to or resistant to dystrophic calcification. Integrative genomics of the so-called Dyscalc locus led to the identification of Abcc6 as the major gene determining dystrophic cardiac calcification.158 Abcc6-deficient mice have soft tissue calcifications develop, like D2-Ahsg−/− mice, albeit they are less extensive in both localization and extent. Interestingly, Abcc6 deficiency is associated with reduced plasma fetuin-A levels and calcifications can be partially corrected by over-expressing fetuin-A,159 suggesting that fetuin-A acts downstream or in concert with Abcc6. Despite heavy early-onset and life-long progressing dystrophic calcification in Abcc6−/− and especially in D2- Ahsg−/− mice, true osteogenesis involving clearly identifiable osteochondrocytic cells has never been reported in these mouse models of calcification. This casts doubt on the now popular hypothesis that pathological calcification is always a form of ectopic osteogenesis. More likely, both genes seem to be involved in systemic mineral homeostasis and transport. Therefore, we have termed fetuin-A a mineral chaperone mediating the solubilization, transport, and elimination from circulation of otherwise insoluble minerals, much like apolipoproteins help in lipid transport and metabolism.104

Because fetuin-A is highly expressed in bone and accounts for 25% of all noncollagen proteins of bone (noncollagenous proteins),102 we examined bone growth and remodeling phenotypes in mixed background 129,B6-Ahsg−/− mice.47 The skeletal structure of these mice appeared normal at birth, but abnormalities were observed in adult 129,B6-Ahsg−/− mice. Maturation of growth plate chondrocytes was impaired, and femurs lengthened more slowly between 3 and 18 months of age. Previously, it had been found that fetuin-A is a soluble TGF-β/BMP-binding protein controlling cytokine access to membrane signaling receptors.155,156 Hence, the altered bone phenotype was explained in terms of failure to block TGF-β–dependent signaling in osteoblastic cells. Mice lacking fetuin-A displayed growth plate defects, increased bone formation with age, and enhanced cytokine-dependent osteogenesis.47

In view of the mineralization regulation effected by fetuin-A, we revisited the bone phenotype of pure-bred B6-Ahsg−/− mice. These mice are unaffected by the secondary hyperparathyroidism and osteoporosis typical of D2-Ahsg−/− mice.103 We essentially reproduced all major findings of the previous study performed in 129,B6-Ahsg−/− mice.47 The mice displayed normal trabecular bone mass in the spine but increased cortical thickness in the femur. Bone composition and mineral and collagen characteristics of cortical bone were unaffected by the absence of fetuin-A. The long bones, especially femora, were severely stunted in B6-Ahsg−/− mice compared to wild-type littermates, resulting in increased biomechanical stability. In addition, we determined increased mineral content in the growth plates of B6-Ahsg−/− long bones, corroborating its physiological role as an inhibitor of excessive mineralization in the growth plate cartilage matrix, a site of vigorous physiological mineralization. We thus demonstrated that growth plate chondrocytes are prone to “pathological calcification” in the absence of fetuin-A, and that active mineralization inhibition is a necessity for proper long bone growth, at least in mice.

The fact that three of the most potent anticalcification compounds, matrix GLA protein,157 pyrophosphate,161 and fetuin-A, all operate in and around growth plate chondrocytes suggests that this cell type is physiologically extremely prone to premature calcification. The pathomechanism of osteogenic vascular calcification likewise critically requires chondrogenic, but not necessarily osteoblastic, differentiation of vascular cells.162–164 It will be interesting to revisit the influence of fetuin-A alone and in combination on chondrocyte mineralization in a way similar to that previously performed with primary osteoblasts10 and smooth muscle

Table 1. Fetuin-A Bioactivities With References

<table>
<thead>
<tr>
<th>Fetuin-A Bioactivity</th>
<th>Biochemical and Cell Culture Work</th>
<th>Animal Study</th>
<th>Clinical/Observational Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase interaction</td>
<td>28–32</td>
<td>34, 35</td>
<td></td>
</tr>
<tr>
<td>Lipid binding</td>
<td>36–39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lectin binding</td>
<td>41–43</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>TGF-β antagonism</td>
<td>45, 46</td>
<td>47, 48</td>
<td></td>
</tr>
<tr>
<td>Antiinflammatory activity</td>
<td>49, 50, 56, 57</td>
<td>52–55</td>
<td></td>
</tr>
<tr>
<td>Insulin receptor antagonism, role in MetS</td>
<td>17, 18, 78</td>
<td>79, 83</td>
<td>70–73</td>
</tr>
</tbody>
</table>


MetS indicates metabolic syndrome; TGF, transforming growth factor.
Clinical Epidemiology of Serum Fetuin-A Levels and Polymorphisms

Approaching the clinical situation in humans and based on the outlined experiments, it is strongly suggested to generally view serum fetuin-A levels in combination with serum albumin as an indicator of nutritional state, as another negative acute phase protein, and as an indicator of overall hepatic protein synthesis, especially in dialysis patients. Patients in advanced stages of chronic kidney disease (CKD) clinically have the most serious cardiovascular and soft tissue calcifications develop, more than any other population, and it is now well-understood that the individual magnitude of calcification significantly corresponds with impaired survival in CKD. Furthermore, CKD patients tend to be in a state of malnutrition and microinflammation or macroinflammation, or both, in which downregulation of proteins such as fetuin-A may be expected.

When the biochemical properties of fetuin-A became more and more apparent, it was a straightforward approach to observe the relationship between serum fetuin-A levels and outcomes in large dialysis populations. In a cohort of >300 hemodialysis patients, the lowest tertile of serum fetuin-A levels was associated with significantly increased all-cause and cardiovascular mortality. Fetuin-A was also found to be inversely correlated with C-reactive protein levels, emphasizing its nature as a negative acute phase reactant. These data were subsequently confirmed, demonstrating mortality risk prediction by fetuin-A deficiency in nearly 300 incident dialysis patients (including patients using peritoneal dialysis). In this study, a specific fetuin-A gene polymorphism (Thr256Ser) was shown to predict particularly low fetuin-A levels and to be associated with an adverse prognosis compared to patients carrying alternative polymorphisms. Hypoalbuminemia was strongly correlated with fetuin-A deficiency, suggesting the expected involvement in the malnutrition-inflammation-atherosclerosis syndrome in clinical fetuin-A deficiency. In a pure cohort of prevalent peritoneal dialysis patients, fetuin-A deficiency was also shown to be linked to features of the malnutrition-inflammation-atherosclerosis syndrome (low albumin, elevated C-reactive protein) and to cardiovascular events and mortality, respectively. Moreover, this study demonstrated an association between low fetuin-A levels and the magnitude of valvular calcification, emphasizing the hypothesized link between progressive calcification and impaired outcomes. In a smaller cohort of hemodialysis patients, a significant association between coronary calcification and fetuin-A deficiency was shown. Taken together, these observations indicate that fetuin-A deficiency may be an important inflammation-related link between cardiovascular calcification and mortality in patients using dialysis.

In patients with normal renal function as well as in predialysis CKD patients, the available information on the relationship between fetuin-A levels, the degree of calcification, and mortality is less clear. Recently, we have shown in the first prospective longitudinal study that low baseline serum levels of fetuin-A are associated with the increase of aortic valve calcification in 77 patients. However, in nearly 1000 patients from the Heart and Soul study focusing on patients at cardiovascular risk, mostly without renal dysfunction, high fetuin-A levels were found to be strongly associated with hyperlipidemia and features of the metabolic syndrome, but not with hard outcome parameters. In patients with diabetes mellitus spanning CKD stages 1 to 4, the magnitude of coronary artery calcification correlated with increased rather than with decreased fetuin-A levels. This finding may be specific for a pure diabetic cohort in which a different pattern of calcium-regulatory systems may be implicated and in which downregulation of fetuin-A may be partially compensated or masked by overnutrition. However, these data may imply that fetuin-A upregulation initially acts as a systemic defense mechanism (early warning system) trying to protect from or counteract against vascular calcifications in their early stages. Such an interpretation is indirectly supported by immunohistochemical findings showing strong fetuin-A deposition, but not synthesis, in areas of vascular calcification and may be better-understood once the metabolism of fetuin-A mineral complexes (calciprotein monomers, calciprotein particles/fetuin–mineral complexes) and concomitant fetuin-A serum depletion is known. A recent study suggested that serum measurements of fetuin-A have to take into account that mineral-bound fetuin-A may compete with free fetuin-A, and that centrifugation sedimentation of serum samples may considerably influence certain assay readouts but not others.

When the calcification burden increases beyond a certain point in chronic long-term CKD, compensatory systems such as fetuin-A release may finally become exhausted and consecutive fetuin-A deficiency may start a vicious cycle of even more progressive extrasosseous calcification and fetuin-A consumption. If this hypothesis of initial adaptive fetuin-A overproduction and later exhaustion of the system is correct, then future research should attempt to identify factors influencing fetuin-A expression and secretion.

Fetuin-A Consumption and Downregulation in Calciphylaxis/Calcific Uremic Arteriolopathy

Perhaps the most intriguing experimental example of fetuin-A consumption was published by Price et al., who demonstrated transient increases of total serum calcium up to 10 mmol/L by high-dose vitamin D treatment in rats within a few hours and a return to normocalcemia within 1 day. A concomitant loss of half the serum fetuin-A that formed a high-molecular-weight fetuin–mineral complex was observed, and it was probably cleared by the reticuloendothelial system or deposited in the bone or in extraskeletal sites. A potential clinical example of fetuin-A consumption and exhaustion may be calciphylaxis (calcific uremic arteriolopathy), a rare but potentially life-threatening syndrome characterized by progressive and painful skin ulcerations associated with media calcification of medium and small cutaneous arterial vessels. Calciphylaxis primarily affects patients using dialysis or after renal transplantation. We reported fetuin-A deficiency with very low serum levels in a case series of calciphylaxis patients. To demonstrate the functional cal-
classification inhibitory capacity of fetuin-A deficiency, we used sera from calcific uremic arteriolopathy patients in an ex vivo \[\text{CaCl}_2\] radioisotope assay, which enables quantification of serum-induced inhibition of a calcium phosphate precipitation. These fetuin-A– deficient calcific uremic arteriolopathy sera were significantly less effective at inhibiting calcium phosphate crystal formation than sera from healthy subjects with appropriate fetuin-A concentrations. This lack of efficacy could be reversed by the addition of purified fetuin-A to the calciphylaxis sera in quantities restoring normal serum levels. In this context, it remained unclear whether calcific uremic arteriolopathy was initially triggered by a lack of fetuin-A in the circulation, whether the system was already exhausted by a major attempt to counteract this fulminant calcification process, or whether levels were low secondary to a calciphylaxis-induced systemic inflammatory reaction.

Figure 6. Calcification-related genes "at work" in vascular smooth muscle cells (VSMC) undergoing metaplasia. After "transdifferentiation" into mineralizing VSMC, the cells elaborate markers of the osteogenic/chondrogenic lineage. The sodium/phosphate cotransporter Pit-1 mediates phosphate transport into the cell. Elevated phosphate levels in the cytoplasm upregulate expression of Runx2/Cbfa-1, an osteogenic transcription factor. In addition, hyperphosphatemia enhances production of apoptotic bodies and matrix vesicles that nucleate vascular mineral deposition. The transforming growth factor (TGF)-β/TGF-like cytokine BMP-7 maintains the contractile phenotype (via Smad 6/similar to mothers against decapentaplegic 6 and Smad 7 signaling) and BMP-2 and TGF-β1 enhance the osteogenic phenotype. Extracellular calcium is transported into matrix vesicles by Ca\(^{2+}\)/channel-forming annexins II, V, and VI. Calcium enhances the phosphate-dependent osteogenic differentiation by upregulation of Pit-1 expression. Pyrophosphate (PP) acts as an inhibitor of basic calcium phosphate crystal growth. The concentration of PP is controlled by nucleotide pyrophosphatase/phosphotransferase-1 (ENPP1), which generates PP, the PP transporter ANK, and tissue-nonspecific alkaline phosphatase (TNAP), which cleaves PP. When cells fail to properly handle a high mineral load because of elevated extracellular calcium phosphate, especially in the absence of extracellular fetuin-A, they will succumb to apoptosis. Apoptotic bodies (AB) containing high amounts of mineral-like matrix vesicles readily mineralize and form a potent nidus for further extracellular matrix calcification. Unlike matrix vesicle-mediated mineralization, AB-mediated mineralization does not require alkaline phosphatase and annexins. In addition, phosphatidylserine (PS) is localized on opposite sides of the plasma membrane of matrix vesicles (inside) and AB (outside). PS is externalized to the outer membrane leaflet during apoptosis. Fetuin-A prevents extracellular and intravesicular basic calcium phosphate growth in matrix vesicles and thus reduces calcium-induced apoptosis in VSMC. BMPR-I indicates BMP receptor-I; MGP, matrix GLA protein; NTP, nucleotide triphosphate; NMP, nucleotide monophosphate; OPN, osteopontin; SM-MHC, smooth muscle myosin heavy chain; TNF-α, tumor necrosis factor-α. Illustration credit: Cosmocyte/Ben Smith.

Conclusions

Although we have focused solely on fetuin-A, we do not advertise fetuin-A as the "holy grail" of mineralization research. We are fully aware that fetuin-A plays its role toward the very end of pathological mineralization, when most of the damage is done and mineralization is imminent that would not occur in the presence of potent mineralization inhibitors like pyrophosphate and matrix GLA protein, and in the absence of local inflammation, cell death, destruction of matrix, and so on. Figure 6 concatenates major principles of pathological mineralization known to date and puts fetuin-A in perspective. Fetuin-A serves as a mineral chaperone, a carrier protein facilitating transport and clearing of potentially proinflammatory and procalcific cargo (waste). The mechanistic and functional analogy between lipoproteins...
and fetuin-A-based calciprotein particles is obvious. No matter what the preferred mechanism of atherosclerotic lesion calcification is, deranged mineral homeostasis, dyslipidemia, compromised scavenging and debris clearing, inflammation, apoptosis, matrix mineralization, and osteogenesis are all known pundits ("partners in crime"), and it seems like fetuin-A counters many of them and thus is a highly pleomorphic protein and is truly a systemic regulator of mineralization.

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Disclosures
None.

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Willi Jahnne-Dechent, Alexander Heiss, Cora Schäfer and Markus Ketteler

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