Calciprotein Particles and Serum Calcification Propensity: Hallmarks of Vascular Calcifications in Patients with Chronic Kidney Disease

Ciprian N. Silaghi 1,*, Tamás Ilyés 1, Adriana J. Van Ballegooijen 2 and Alexandra M. Crăciun 1,*

1 Department of Molecular Sciences, University of Medicine and Pharmacy “Iuliu Hatieganu”,
400012 Cluj-Napoca, Romania; tamasilyes94@gmail.com (T.I.); acraciun@umfcluj.ro (A.M.C.)
2 Department of Nephrology & Epidemiology and Biostatistics, Amsterdam UMC, location VUmc,
1117 HV Amsterdam, Netherlands; aj.vanballegooijen@vumc.nl

* Correspondence: silaghi.ciprian@umfcluj.ro

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Abstract: Cardiovascular complications are one of the leading causes of mortality worldwide and are strongly associated with atherosclerosis and vascular calcification (VC). Patients with chronic kidney disease (CKD) have a higher prevalence of VC as renal function declines, which will result in increased mortality. Serum calciprotein particles (CPPs) are colloidal nanoparticles that have a prominent role in the initiation and progression of VC. The T50 test is a novel test that measures the conversion of primary to secondary calciprotein particles indicating the tendency of serum to calcify. Therefore, we accomplished a comprehensive review as the first integrated approach to clarify fundamental aspects that influence serum CPP levels and T50, and to explore the effects of CPP and calcification propensity on various chronic disease outcomes. In addition, new topics were raised regarding possible clinical uses of T50 in the assessment of VC, particularly in patients with CKD, including possible opportunities in VC management. The relationships between serum calcification propensity and cardiovascular and all-cause mortality were also addressed. The review is the outcome of a comprehensive search on available literature and could open new directions to control VC.

Keywords: calciprotein particles; calcification propensity; chronic kidney disease; vascular calcification

1. Introduction

Serum calciprotein particles (CPPs) are colloidal nanoparticles comprising a combination of proteins (mainly fetuin-A, but also albumin and Gla-rich protein (GRP)) and calcium (Ca2+) containing compounds, primarily calcium phosphate [1–3]. They are first formed by the binding of Ca2+ precursors to the acidic residues of fetuin-A, a glycoprotein secreted by the liver [1,4]. These calcium–protein complexes, also known as calciprotein monomers, pass through further aggregation and maturation, resulting in primary calciprotein particles (CPP I) and later on, secondary calciprotein particles (CPP II) [5–7]. CPP I are small spherical colloidal nanoparticles that contain amorphous calcium phosphate, while CPP II contain crystalline calcium phosphate at their core, are larger than CPP I, and have a needle-shaped structure. This transition from CPP I to CPP II is called “ripening” and is hypothesized to be attributed to a reorganization of the colloidal nanoparticles into a more stable form [5]. The ripening process is influenced by a number of factors such as the concentration of fetuin-A, Ca2+, magnesium (Mg2+), phosphate (Pi), as well as the temperature and pH of the surrounding microenvironment [1,6,8].
The transition from CPP I to CPP II, which takes place naturally in serum, can also be induced in vitro, and the time needed for the transition to take place can be measured. Half of the time needed for the spontaneous transition from CPP I to CPP II, designated as $T_{50}$, has been established as a strong predictor of the calcifying properties of serum [9]. A higher $T_{50}$ is beneficial since serum with a higher $T_{50}$ is less prone to calcify tissues compared to serum that has a lower $T_{50}$.

Vascular calcification (VC) results in the thickening and increased rigidity of muscular arterial walls [10]. This is the consequence of two main types of calcification: intimal and medial calcification. Intimal calcification is associated with atherosclerosis, $\text{Ca}^{2+}$ being deposited along with lipoproteins as well as phospholipids [11,12]. Medial calcification, which is more prevalent in chronic kidney disease (CKD), is the result of an osteogenic process similar to intramembranous ossification, which is independent of atherosclerosis and causes a decrease in compliance of the vessel wall [13–15]. Medial calcification occurs earlier in CKD patients compared to the general population [16].

With respect to CPPs in general and $T_{50}$ in particular, there have been no reviews published until now that summarize findings related to both CPPs and $T_{50}$. Therefore, the purpose of this review was to offer a synopsis of all studies published on CPPs and $T_{50}$, respectively. We also aim to analyse and discuss their roles and clinical significance in patients prone to developing VC, as well as to establish possible new directions in the management of VC.

2. Methodology

2.1. Search Strategy

All databases that could be accessed through the PubMed search engine were selected for this review. Human, animal, and in vitro studies were all taken into account. Due to the specific nature of the selected domain and the fact that the majority of research papers were published relatively recently, the period of publication was not limited. A set of search terms was selected as follows: “Calciprotein particles”, “$T_{50}$ AND calcification”, “Serum calcification propensity”. The search was performed in PubMed on the 4th of January 2020 for both search strings, yielding a total of 162 studies (78, 30, and 54 results, respectively). The results of the searches were organized into lists that were cross-checked between search terms, with duplicates being eliminated. After the initial screening of titles and abstracts, full-text articles were obtained for all eligible studies.

2.2. Selection, Screening, and Inclusion

The authors jointly selected the inclusion and exclusion criteria. Only articles with abstracts were selected for screening, written in English including human, animal, and in vitro studies.

Studies that did not address CPPs and/or $T_{50}$ in a medically relevant manner, such as physical or chemical characterization of CPPs, and studies that lacked a clear definition of methods and materials were not included. Reviews and case reports were excluded as well.

The identification, selection, screening, and inclusion process is summarized in Figure 1. After cross-checking and eliminating duplicates, the results of the search string “Serum calcification propensity” yielded three studies that were subsequently included in the same category as $T_{50}$. In total, 18 studies were included for CPPs [3,17–33] and 30, including the aforementioned 3 studies, for $T_{50}$ [34–63].
3. Molecular Background

3.1. Fetuin-A and Calciprotein Particles

While CPPs contain a number of proteins that can bind Ca\(^{2+}\), e.g., Gla-rich protein (GRP) [3], as well as other serum proteins and lipoproteins such as albumin and apolipoprotein A1 [2], the main protein within the CPP structure is fetuin-A, also known as alpha-2-HS-glycoprotein. It is a 55–60 kDa glycoprotein, synthesized and secreted by the liver, which undergoes post-translational modifications, including phosphorylation [4,64]. While phosphorylation is crucial for its various interactions, e.g., with the insulin receptor, it is not required for mineral binding due to the number of acidic residues [1,4,65,66]. Each molecule of fetuin-A can bind up to 6 Ca\(^{2+}\) ions [67]. Calcium and Pi bound by fetuin-A form protein–mineral complexes called calciprotein monomers, the aggregation of which results in the formation of plasma-soluble amorphous colloidal particles, referred to as CPP I. The CPP I, which is spherical in nature and has a diameter of around 75 nm, circulates in plasma and eventually undergoes rearrangement into CPP II, which is more dense, with a larger diameter (120 nm), insoluble in serum, and has a needle-shaped crystalline structure [1]. This transition from the primary, more unstable form, to the secondary, more stable form, is dubbed “ripening” [5]. The process is illustrated in Figure 2.

CPP I and CPP II are cleared by macrophages, especially Kupffer cells in the liver, thereby preventing tissue deposition of Ca\(^{2+}\) and Pi [68]. Studies have shown that CPP II induces vascular smooth muscle cell (VSMC) calcification in vitro, as well as the secretion of tumour necrosis factor α (TNF-α) in macrophages, while CPP I does not. CPP II was found to increase bone morphogenetic protein-2 as well as nuclear factor kappa-B expression in VSMCs. The calcification of VSMCs was
also shown to be the result of the cellular uptake of CPP II, with CPP II being detected intracellularly in calcified VSMCs [29]. Both CPP I and CPP II were found to induce VSMC intimal hyperplasia, which was more pronounced in the case of CPP II [18]. Moreover, CPPs were found to induce secretion of interleukin 1β (IL-1β) in macrophages, however, to a lesser degree than hydroxyapatite crystals [31]. While both forms of CPP have pro-inflammatory effects, it is still less prominent than crystalline hydroxyapatite. The more pronounced pro-inflammatory effect of CPP II compared to that of CPP I might be attributed to its content of hydroxyapatite in crystalline form.

**Figure 2.** Fetuin-A transformation into CPP II. Abbreviations: Pi, phosphate; CPP I, primary calciprotein particle; CPP II, secondary calciprotein particle.

The CPPs are detected and quantified in serum indirectly, by assessing the fetuin-A levels via enzyme-linked immunosorbent assay (ELISA), before and after a high-speed centrifugation that precipitates all CPPs as CPP II. The difference between fetuin-A concentrations before and after centrifugation is interpreted as the amount of CPPs in the serum sample [33,69]. Because this method induces the ripening process before measuring CPP content, it only brings information regarding the total concentration of CPPs, without differentiating between CPP I and CPP II. To measure CPP I and CPP II concentrations independently, a flow-cytometry method can be used [70].

### 3.2. Calcifying Properties of Serum

A method for measuring the calcification inhibition capacity of serum was elaborated by Ismail et al. [71] based on electrochemical impedance. A prototype probe was successfully used to measure the impedance of a test solution consisting of bovine albumin, Ca^{2+}, and Pi. Upon the addition of a calcification inhibitor, in that case fetuin-A, the electrical impedance of the solution would increase proportionately to the Ca^{2+} content, due to the inhibitor consuming Ca^{2+} ions by forming CPP I. Thus, the calcification inhibition capacity of the serum could be determined by measuring the variation of impedance of a solution containing Ca^{2+} and Pi in a known concentration, after the addition of serum.

Pasch et al. [9] were the first to develop a plate-based nephelometric assay to measure the time needed for the transition from CPP I to CPP II in serum treated with Ca^{2+} and Pi solutions, and proposed the use of one half of the transition time to maximum turbidity, also known as T_{50}, as a parameter to describe the calcifying properties of serum. The influence of factors such as pH and concentrations of various serum constituents upon T_{50} was also analysed, and is summarized in Figure 3.
4. Results

4.1. Calciprotein Particles

Human studies on serum CPP levels are summarized in Table 1, animal and in vitro studies on CPP are summarized in Table 2. The majority of studies used detection methods that did not differentiate between the two types of CPP. To avoid confusion, we used the term total CPP (tCPP) when referring to studies that did not specify the type of CPP analysed.

Table 1. Summary of 11 human studies on calciprotein particle (CPP).

| Author et al., Year | Study Design, Duration | Number of Subjects, Disease | CPP Type Studied | Findings |
|---------------------|------------------------|-----------------------------|-----------------|----------|
| Nakazato et al. 2019 [20] | cross-sectional, N/A | 71 ACS | tCPP | High CPP levels associated with atherosclerosis. |
| Chen et al. 2019 [23] | cross-sectional, N/A | 45 CKD stage IV-V | CPP II | Larger CPP II diameter in patients with VC. |
| Viegas et al. 2018 [3] | cross-sectional, N/A | 16 CKD stage II-IV, 20 CKD stage V | tCPP | CPP from CKD stage V patients contained less fetuin-A and GRP and had CPP II like characteristics. |
| Yamada et al. 2018 [28] | cross-sectional, N/A | 10 diabetes mellitus type 2 | tCPP | CPP elevated 2 h post-meal, CPP inversely correlated with eGFR. |
| Cai et al. 2015 [30] | cross-sectional, N/A | 20 peritoneal dialysis | tCPP | CPP present, fetuin-A abundant in peritoneal dialysis effluent. |
| Smith et al. 2013 [32] | cross-sectional, N/A | 11 CKD stage III-IV, 42 HD, 18 peritoneal dialysis, 13 chronic inflammatory disease | tCPP | CPP increased in HD patients with calcific uremic arteriolopathy. |
| Smith et al. 2012 [33] | cross-sectional, N/A | 200 CKD stage III-IV | tCPP | Higher CPP levels associated with increased aortic stiffness. |
| Cai et al. 2018 [24] | prospective cohort, 7 weeks | 12 peritoneal dialysis | tCPP | Dialysate with higher Ca\(^{2+}\) concentration had higher CPP content. |
| Ruderman et al. 2018 [25] | prospective cohort, 12 months | 62 HD | CPP I | Increase of serum CPP I after cessation of cinacalcet treatment. |
| Bressendorff et al. 2019 [17] | Interventional, 28 days | 57 HD | CPP I, CPP II | Higher Mg\(^{2+}\) concentration dialysis solution reduced both CPP I and CPP II levels, compared to standard dialysis solution. |
| Nakamura et al. 2019 [21] | Interventional, 16 weeks | 24 HD | tCPP | Lower CPP in lanthanum carbonate treated patients vs. calcium carbonate. |

Abbreviations: HD, haemodialysis; ACS, acute coronary syndrome; CPP, calciprotein particle; CPP I, primary calciprotein particle; CPP II, secondary calciprotein particle; tCPP, total calciprotein particles; CKD, chronic kidney disease; VC, vascular calcification; GRP, Gla-rich protein; eGFR, estimated glomerular filtration rate; N/A, not applicable.
Table 2. Summary of 1 animal and 6 in vitro studies on CPP.

| Author, Year          | Study Design | Animals/Cells | CPP Type Studied | Findings                                                                 |
|-----------------------|--------------|---------------|------------------|---------------------------------------------------------------------------|
| Nemoto et al. 2019    | animal       | rats with 5/6 nephrectomy | tCPP             | Lower CPP in rats treated with sucroferric oxyhydroxide. Both CPP I and CPP II induced VSMC intimal hyperplasia, more pronounced in case of CPP II. |
| Shishkova et al. 2019 | in vitro     | VSMCs         | CPP I, CPP II    | Both CPP I and CPP II induced VSMC intimal hyperplasia, more pronounced in case of CPP II. |
| Ter Braake et al. 2019| in vitro     | VSMCs         | CPP II           | CPP II induced VSMC calcification. H2S inhibits CPP induced VSMC calcification. |
| Aghagolzadeh et al. 2017| in vitro      | VSMCs         | tCPP             | Pi or CPP II alone did not initiate VSMC mineralization, but CPP II with Pi did. CPP II induced calcification in VSMCs, CPP I did not. CPP induce secretion of TNF-α and IL-1β in macrophages, with a more pronounced effect being attributed to CPP II. This pro-inflammatory response, however, was still inferior to that induced by pure hydroxyapatite crystals. |
| Cai et al. 2017       | in vitro     | VSMCs         | CPP II           | Pi or CPP II alone did not initiate VSMC mineralization, but CPP II with Pi did. CPP II induced calcification in VSMCs, CPP I did not. CPP induce secretion of TNF-α and IL-1β in macrophages, with a more pronounced effect being attributed to CPP II. This pro-inflammatory response, however, was still inferior to that induced by pure hydroxyapatite crystals. |
| Aghagolzadeh et al. 2016| in vitro      | VSMCs         | CPP I, CPP II    | CPP II induced calcification in VSMCs, CPP I did not. CPP induce secretion of TNF-α and IL-1β in macrophages, with a more pronounced effect being attributed to CPP II. This pro-inflammatory response, however, was still inferior to that induced by pure hydroxyapatite crystals. |
| Smith et al. 2013     | in vitro     | VSMCs         | tCPP             | Pi or CPP II alone did not initiate VSMC mineralization, but CPP II with Pi did. CPP II induced calcification in VSMCs, CPP I did not. CPP induce secretion of TNF-α and IL-1β in macrophages, with a more pronounced effect being attributed to CPP II. This pro-inflammatory response, however, was still inferior to that induced by pure hydroxyapatite crystals. |

Abbreviations: VSMCs, vascular smooth muscle cells; CPP, calciprotein particle; CPP I, primary calciprotein particle; CPP II, secondary calciprotein particle; tCPP, total calciprotein particles; H2S, hydrogen sulphide; Pi, phosphate; TNF-α, tumour necrosis factor α; IL-1β, interleukin 1β.

Dialysate from haemodialysis (HD) patients was found to contain CPP, and higher dialysate Ca2+ content was found to be associated with higher CPP concentration [24,30]. This suggests that CPP can be cleared from the plasma of patients with chronic kidney disease (CKD) through HD. In addition, CPP were found to induce VSMC calcification and intimal hyperplasia, with higher serum levels of CPP being associated with increased aortic stiffness [18,26,33]. CPP also induced the secretion of TNF-α and IL-1β in macrophages, with a more pronounced effect being attributed to CPP II. This pro-inflammatory response, however, was still inferior to that induced by pure hydroxyapatite crystals [29,31].

4.2. Calcification Propensity

Observational studies of T50 and outcomes are summarized in Table 3, and human intervention studies are summarized in Table 4. The majority of studies included in this section concern T50 in CKD and/or kidney transplant patients.

Oral Mg2+ supplementation, as well as increased Mg2+ concentration in dialysis solution was found to increase T50 in CKD patients [39,45,51]. The T50 was also found to be associated with serum Mg2+ levels in CKD patients, but not with eGFR [50]. Serum Mg2+ levels were directly associated with T50, which suggests that both oral Mg2+ supplementation, as well as increasing the Mg2+ content of dialysis solution could be a viable method to counterbalance VC to some extent in CKD patients. The use of citrate-buffered dialysis solution was found to significantly increase T50 as opposed to standard acetate-buffered dialysis solution in HD patients [34,46]. While platelet derived growth factor B hypomorphic animal brains showed signs of calcification, T50 did not differ compared to controls [61]. Lower T50 levels were also found to be associated with lower tissue oxygenation, as well as an increase in all-cause and cardiovascular mortality, especially in CKD and kidney transplant patients [36,42,48,49,52,56,57,60].
Table 3. Summary of 18 observational studies on T<sub>50</sub> and health outcomes.

| Author, Year | Study Design | Follow-Up Time | Number of Subjects, Disease | Findings |
|--------------|--------------|----------------|----------------------------|----------|
| Bullen et al. 2019 [41] | cross-sectional | N/A | 149 men with osteoporosis | T<sub>50</sub> was not associated with bone mineral density. |
| Dahdal et al. 2018 [47] | cross-sectional | N/A | 168, SLE | T<sub>50</sub> was negatively associated with disease activity. |
| Pruijm et al. 2017 [48] | cross-sectional | N/A | 58, CKD; 48, hypertension | Lower T<sub>50</sub> was associated with reduced tissue oxygenation and perfusion. |
| Bielez et al. 2017 [50] | cross-sectional | N/A | 118, CKD stage I–V | T<sub>50</sub> associated with Pi, Mg<sup>2+</sup> and fetuin-A but not with eGFR. |
| Dekker et al. 2016 [54] | cross-sectional | N/A | 64, HD | T<sub>50</sub> increased post-haemodialysis and post-haemodiafiltration. |
| Voelkl et al. 2018 [63] | cross-sectional | N/A | 16, CKD; 20, HD | T<sub>50</sub> was lower in CKD patients compared to controls. |
| van Dijk et al. 2019 [35] | prospective cohort | 15 years | 216, type 1 diabetes | T<sub>50</sub> not associated with mortality. |
| Bundy et al. 2019 [36] | prospective cohort | At TOD or 11.2 years | 3404, CKD stage II–IV | Lower T<sub>50</sub> associated with cardiovascular events and all-cause mortality. |
| Ponte et al. 2019 [37] | prospective cohort | 3 months | 46, HD; 12, peritoneal dialysis | Higher T<sub>50</sub> after dialysis initiation. |
| Bundy et al. 2019 [38] | prospective cohort | 3.2 ± 0.6 years | 780, CKD stage II–IV | Lower T<sub>50</sub> was associated with greater CAC severity and progression, however, T<sub>50</sub> was not associated with CAC incidence. |
| Bostom et al. 2018 [42] | prospective cohort | median of 2.18 years | 685, CVD | Lower T<sub>50</sub> and fetuin-A levels were associated with greater risk for CVD outcomes. |
| Pasch et al. 2017 [49] | prospective cohort | At TOD or first non-fatal CVE | 2785, HD | Lower T<sub>50</sub> associated with all-cause mortality, myocardial infarction, and peripheral vascular events. |
| Lorenz et al. 2017 [52] | prospective cohort | 24 months | 188, HD | T<sub>50</sub> rate of decline significantly predicted all-cause and cardiovascular mortality. |
| Dahle et al. 2016 [56] | prospective cohort | median of 5.1 years | 1435, kidney transplant | Lower T<sub>50</sub> associated with all-cause and cardiac mortality. |
| Keyzer et al. 2016 [57] | prospective cohort | median of 3.1 years | 699, kidney transplant | Lower T<sub>50</sub> associated with increased graft failure, all-cause, and cardiac mortality. |
| de Seigneux et al. 2015 [59] | prospective cohort | 1 year | 21, kidney donors | T<sub>50</sub> was independent of eGFR. |
| Smith et al. 2014 [60] | prospective cohort | median of 5.3 years | 184, CKD stage III–IV | Lower T<sub>50</sub> associated with higher all-cause mortality. |
| Berchtold et al. 2016 [58] | prospective cohort | between 2 and 43 years | 129, kidney transplant | T<sub>50</sub> associated with interstitial fibrosis and vascular lesions. |

Abbreviations: SLE, systemic lupus erythematosus; HD, haemodialysis; CKD, chronic kidney disease; CAC, coronary artery calcification; CVD, cardiovascular disease; Mg<sup>2+</sup>, magnesium; TOD, time of death; CVE, cardiovascular event; Pi, phosphate; eGFR, estimated glomerular filtration rate; N/A, not applicable.
### Table 4. Summary of 11 human interventional studies on T₅₀ with health outcomes.

| Author, Year       | Study Duration | Number of Subjects, Disease | Findings                                                                 |
|--------------------|----------------|-----------------------------|--------------------------------------------------------------------------|
| Smerud et al. 2017 | 1 year         | 123, kidney transplant      | T₅₀ increased with no further change after 1 year, ibandronate had no effect on T₅₀. |
| Andrews et al. 2018| 12 weeks       | 80, CKD with hyperuricemia  | Allopurinol lowered uric acid levels but had no effect on T₅₀. Acetate-free, citrate-acidified, standard bicarbonate dialysis solution increased T₅₀ compared to acetate dialysis solution. |
| Lorenz et al. 2018 | 3 months       | 78, HD                      | Paricalcitol supplementation had no effect on T₅₀.                       |
| Ussif et al. 2018  | 1 year         | 76, kidney transplant       | Higher dialysis solution Mg²⁺ concentration increased T₅₀.                |
| Bressendorff et al.2018 | 28 days   | 57, HD                      | Insignificant decrease of T₅₀ in the group treated with oral calcium carbonate supplement. |
| Bristow et al. 2016 | 3 months       | 41, post-menopausal women   | Oral Mg²⁺ supplementation increased T₅₀.                                 |
| Bressendorff et al.2017 | 8 weeks   | 36, CKD III–IV              | Oral bicarbonate supplementation showed no effect on T₅₀ in acidotic CKD patients. |
| Aigner et al. 2019 | 4 weeks        | 35, CKD                     | Oral sodium bicarbonate supplementation showed no effect on T₅₀ in CKD patients with low serum bicarbonate levels. |
| Kendrick et al. 2018| 14 weeks       | 18, CKD                     | Citric acid-buffered dialysis solution increased T₅₀ compared to acetate-buffered solution. |
| Ter Meulen et al. 2019[34] | 2 weeks  | 18, HD                      | Effervescent, oral, calcium-magnesium citrate increased T₅₀.               |
| Quiñones et al. 2019 | 2 weeks     | 9, CKD stage III, 9, CKD stage V |                                                                 |

Abbreviations: HD, haemodialysis; CKD, chronic kidney disease; Mg²⁺, magnesium.

5. Discussion

This comprehensive review showed that multiple lines of evidence (cell, animal, and human) indicate that T₅₀ is shorter in CKD and dialysis populations. A large amount of studies indicate that a lower T₅₀ is related to VC, cardiovascular events, and mortality. These findings are robust across various populations and open up new directions to modify VC especially in patients with CKD. One of these factors that can influence the tendency to calcify is Mg²⁺. Oral Mg²⁺ supplementation as well as increased dialysis solution Mg²⁺ concentration had beneficial effects on T₅₀ [39,45,52], and a lower T₅₀ was associated with cardiovascular and all-cause mortality in various populations [36,42,49,52,56,57,60]. It is worth noting the correlation between higher serum CPP content, especially CPP II, and VSMC inflammation as well as calcification [18,26,29,31,33]. Taking the included studies into consideration, we address two topics for further research in this relatively recent domain.

5.1. The Effect of Dialysis Solution Composition upon Serum Calcification Propensity in CKD Patients

The transition from CPP I to CPP II is delayed by the presence of Mg²⁺, this effect being dependent upon the concentration of Mg²⁺. The presence of Mg²⁺, however, does not inhibit VSMC calcification in the presence of CPP II, suggesting that the anti-calcific effects of Mg²⁺ are more related to preventing the transition from CPP I to CPP II [19]. This would also explain the effect of Mg²⁺ upon increasing T₅₀. However, the exact mechanism by which Mg²⁺ inhibits the maturation of CPP I is not completely understood. One possible mechanism might lie in the ability of Mg²⁺ to inhibit Ca²⁺ and Pi crystallization [72], which is a necessary step in CPP maturation.
Studies suggest that there is a significant amount of CPPs in the dialysate of CKD patients on peritoneal dialysis. That CPP content was also directly proportional to the dialysate’s Ca\(^{2+}\) content [24]. While HD was found to increase T\(_{50}\), thus reducing the calcification propensity of the patient’s plasma [37,54], serum CPP I and CPP II levels seem to be unaffected by standard HD [17].

First of all, this would suggest that the increase in T\(_{50}\) after initiation of HD is not attributed to the clearance of CPPs per se, but to the reduction of factors that precipitate the ripening process, most probably the reduction of Ca\(^{2+}\) and Pi. Second of all, CPPs, while not being cleared from the serum under standard HD conditions, are cleared by peritoneal dialysis to some degree. However, if the Mg\(^{2+}\) concentration of HD dialysis solution is increased, CPPs appear to pass the dialysis membrane and are cleared from the patient’s serum [17]. This would, in part, explain the significant increase of T\(_{50}\) in patients treated with a dialysis solution containing a larger Mg\(^{2+}\) concentration compared to standard solution [45].

In addition to the beneficial effect of increased Mg\(^{2+}\) content in dialysis solution upon the serum calcification propensity in CKD patients, the use of an acetate-free, citrate-acidified dialysis solution was also found to increase T\(_{50}\) thus reducing the calcification propensity [34,46].

Patients with CKD who received oral Mg\(^{2+}\) supplementation showed a significant increase in T\(_{50}\) [39,51]. In post-menopausal women, the introduction of oral Ca\(^{2+}\) supplementation showed a decrease in T\(_{50}\), however, this decrease did not differ significantly from the control group [55]. These observations correspond with the findings of Pasch et al. [9], who determined that higher serum Mg\(^{2+}\) levels will increase T\(_{50}\). A summary of the aforementioned factors upon T\(_{50}\) is presented in Figure 4.

![Figure 4](image_url)

**Figure 4.** Summary of factors that increase T\(_{50}\) in CKD patients. Abbreviations: Mg, magnesium; VC, vascular calcification.

Furthermore, it is well known that patients with CKD have a significantly higher risk for VC and associated cardiovascular mortality [73]. Developing a standardized treatment plan for end-stage CKD patients on HD or peritoneal dialysis that would take into account the above outlined criteria, namely the increased Mg\(^{2+}\) content of dialysis solution and the use of citrate instead of acetate, should be validated and subsequently introduced into a therapeutic protocol. Patients with HD, as well as those with CKD who do not require HD, could also benefit from a reduction in oral Ca\(^{2+}\) and an increase in oral Mg\(^{2+}\) supplementation, respectively. Such an approach to the management of VC and the possible ensuing reduction of cardiovascular mortality rates in CKD patients could lead to an increased quality of life, especially for patients undergoing HD or peritoneal dialysis, delaying the onset or decreasing the severity of cardiovascular complications associated with CKD.

### 5.2. The T\(_{50}\) Test Could Be Used as a Factor in the Staging and/or Prognosis of CKD

There are plentiful studies, conducted on large sample sizes, that came to the conclusion that lower T\(_{50}\) corresponding to higher calcification propensity is strongly associated with increased cardiovascular and all-cause mortality rates [36,42,49,52,56,57,60]. Lower T\(_{50}\) was also associated with coronary artery calcification progression as well as greater risk for cardiovascular disease outcomes, such as myocardial infarction and peripheral vascular events [38,42,49].
The investigation of a possible association between $T_{50}$ and eGFR could lead to the development of a reference interval for $T_{50}$ in CKD patients, which is dependent on CKD stage. Such a reference interval, which has not yet been established, could be used as an additional prognostic parameter for CKD patients, especially those undergoing HD or peritoneal dialysis treatment. There was conflicting evidence that links serum CPP levels and $T_{50}$ to eGFR. Yamada et al. [28] found that CPP levels were inversely associated with eGFR in diabetic patients. However, that study was conducted on diabetic patients, not CKD patients, and the patient group was relatively small as well. On the other hand, Bielesz et al. [50], found that $T_{50}$ was not associated with eGFR in CKD stage I–V patients, instead being associated with numerous parameters, including Pi and Mg$^{2+}$ levels. A similar result was obtained by de Seigneux et al. [59], who discovered that $T_{50}$ was independent of eGFR in kidney transplant donors, which could be attributed to the compensation effect of an otherwise healthy remaining kidney. Those studies clearly pointed that while serum CPP levels are correlated with eGFR, $T_{50}$ was not.

The CPP levels and $T_{50}$ do not seem to be directly correlated with one another, although $T_{50}$ is greatly influenced by serum Ca$^{2+}$ levels and, in addition, CPP levels are directly proportional to circulating Ca$^{2+}$ levels. Considering the previously discussed ideas, it could be hypothesized that CPP levels are correlated with $T_{50}$, justifying further studies in larger populations to investigate the association between $T_{50}$ and eGFR. However, until the completion of this review, no studies have identified this relationship.

Even in the absence of a link between $T_{50}$ and eGFR, but in the context of association between higher serum calcification propensity and increased cardiovascular and all-cause mortality rates especially in CKD patients, the use of $T_{50}$ as risk factor that can be monitored should be considered. The ensuing introduction of measures to decrease calcification propensity could significantly reduce VC and related mortality in CKD patients.

An interesting opportunity would be to expand the area of research towards the involvement of CPPs in the calcification paradox, in which the presence of vascular calcification overlaps at the same time with bone demineralization assessed by a decrease in bone mineral density (BMD) [74]. It is difficult to decode how CPP and the interplay between vasculature–bone–kidney underlie the deleterious effect of calcification. On one hand, fetuin-A accumulates in calcified atherosclerotic plaques [75], but also in bone where it inhibits mineralization and halts bone matrix protein expression [76]. On the other hand, serum levels of fetuin-A were found to be decreased in patients with end-stage renal disease [77]. Contrariwise, serum CPP increases in patients with CKD III–IV, with it being the highest in HD patients [32] but with less fetuin-A content as CKD stage worsens [3]. Probably the turn-over of CPP is accelerated in CKD patients, but fetuin-A is consumed exerting its systemic anti-calcification effect necessary to counteract VC as CKD stage aggravates.

In addition to the well-known presence of VC in patients with CKD, an important decrease of BMD was also reported [78]. In maintenance HD patients, serum fetuin-A was inversely associated with coronary artery calcification and positively with BMD [79]. In respect to VC, serum CPP appears to behave divergently regarding fetuin-A dynamics in CKD patients: higher CPP levels are associated with increased aortic stiffness [33] and larger CPP II diameters were found in patients with VC [23]. As might be expected, $T_{50}$ was inversely associated with coronary artery calcification (CAC) severity in CKD patients [38], thereby, the $T_{50}$ test seems to mimic serum fetuin-A variations in respect to VC, as they were found to be associated [50]. Regarding the loss of skeletal mineral, $T_{50}$ was not associated with BMD [38] and in the case of CPPs we did not find conclusive studies. To make the puzzle even more complicated, we could introduce the relationship between CPPs or $T_{50}$ and eGFR, as discussed above. Thereby, CPPs were found to be inversely correlated with eGFR [28], instead of $T_{50}$, which was independent of eGFR [50,59].

However, an attempt to explain the paradox of calcification on the vasculature–bone–kidney axis only in terms of fetuin-A content of CPPs is an exercise of simplification. Given this standpoint, more targeted studies are needed to demonstrate that CPPs are more likely to hold the key on how physiological ossification has correspondence with pathological calcification.
Nevertheless, we need to take into account the current limitations of the $T_{50}$ test. Several weaknesses were identified by Pasch et al. [9]: the test overrides the contribution of VSMCs and calcifying myeloid cells in promoting VC in vivo, and the serum pH had no influence on the test due to the presence of a strong buffer. Another issue is attaining standardized conditions to perform the test. Consequently, even if a reference interval would be preferable to be established, each laboratory is likely to set up its own different reference interval, hence it is hard to envisage an accepted consensus interval. The test is robust but needs further development in terms of time per test which is too long to be clinically implemented for the moment: to perform a 96-well format takes 10 h [9].

In addition, simply minimizing the $T_{50}$ as a marker only for VC may be incomplete. The $T_{50}$ could be considered as a momentary status of the sum of pro- and anti-calcification factors in the serum of a patient, but this may have implications on other pathophysiological processes, thus opening a wide field of research. Accordingly, the term mineral stress has been coined by Pasch et al. [80] and refers to the interaction between inflammation, oxidative stress, and calcification promoted by CPP II.

6. Conclusions

The relatively recent discovery of CPPs opens up new possibilities for the prevention of VC and the attempt to quantify the serum calcification propensity via $T_{50}$. Even though the factors that influence serum CPP levels, including their ripening process, as well the effect of various factors upon $T_{50}$ and its variation in different diseases is incompletely understood, there is mounting evidence suggesting that $T_{50}$ could be a viable marker in the assessment of VC. Moreover, $T_{50}$ could be valuable in managing VC in CKD patients, especially those undergoing HD, who have a significantly increased risk for developing cardiovascular complications. In these situations, the early introduction of a treatment strategy that increases $T_{50}$ could mitigate the obvious complications related to VC. Such an approach is still at an early phase, warranting future studies on the use of $T_{50}$ as a standard tool in the assessment of VC, thus allowing early measures to prevent cardiovascular complications in patients at risk.

Abbreviations:

- **BMD** Bone Mineral Density
- **$Ca^{2+}$** Calcium
- **CAC** Coronary Artery Calcification
- **CKD** Chronic Kidney Disease
- **CPP I** Primary Calciprotein Particles
- **CPP II** Secondary Calciprotein Particles
- **CPP** Calciprotein Particles
- **CVD** Cardiovascular Disease
- **CVE** Cardiovascular Event
- **eGFR** Estimated Glomerular Filtration Rate
- **ELISA** Enzyme-Linked Immunosorbent Assay
- **GRP** Gla-Rich Protein
- **HD** Haemodialysis
- **$H_2S$** Hydrogen Sulphide
- **IL-1β** Interleukin 1β
- **$Mg^{2+}$** Magnesium
- **N/A** Not Applicable
- **Pi** Phosphate
- **SLE** Systemic Lupus Erythematosus
- **tCPP** Total Calciprotein Particles
- **TNF-α** Tumour Necrosis Factor α
- **TOD** Time of Death
- **VC** Vascular Calcification
- **VSMC** Vascular Smooth Muscle Cell
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