Complement modulation in renal replacement therapy

Poppelaars, Felix

Chapter 4

Intradialytic complement activation precedes the development of cardiovascular events in hemodialysis patients.

Felix Poppelaars
Mariana Gaya da Costa
Bernardo Faria
Stefan P. Berger
Solmaz Assa
Anita H. Meter-Arkema
Mohamed R. Daha
Willem J. van Son
Casper F.M. Franssen
Marc A.J. Seelen

Submitted

Abstract

Background

Hemodialysis (HD) is a life-saving treatment for patients with end stage renal disease. However, HD patients have increased rates of cardiovascular morbidity and mortality. Previously, a link between the complement system and cardiovascular events (CV-events) has been reported. In HD, systemic complement activation occurs due to blood-to-membrane interaction. We hypothesize that complement activation together with inflammation and thrombosis are involved in the development of CV-events in HD patients.

Methods

Plasma samples were collected from 55 patients at different time points during one HD session. Plasma levels of MBL, properdin and C3d/C3 (as a measure of complement activation) were assessed by ELISA. In addition, levels of vWF, TNF-α, and IL-6/IL-10 ratios were determined. HD patients were followed for the occurrences of CV-event during a maximum follow-up of 45 months.

Results

During median follow-up of 32 months, 17 participants developed CV-events. In the CV-event group, the C3d/C3-ratio peaked 30 minutes after the start of a HD session, while in the event-free group the ratio increased only mildly. In accordance, HD patients that develop a CV-event also had a sustained higher IL-6/IL-10-ratio at the start of the HD session, followed by a rise in TNF-α levels and vWF at the end of the session.

Conclusions

In conclusion, these findings suggest that HD-induced complement activation is predominantly evident in the patients that will develop a CV-event. In addition, in these patients complement activation is accompanied by a pro-inflammatory and pro-thrombotic response. Therefore, our data suggest that complement, inflammation, and coagulation are involved in the increased CV risk of HD patients.

Introduction

Renal replacement therapy (RRT) represents a cornerstone in the treatment of patients with end stage renal disease (ESRD). Hemodialysis (HD) remains the most common form of RRT.[1] Despite being lifesaving, HD comes with a risk.[2] The life expectancy and quality of life of patients on dialysis is inferior to the general population. Overall, HD has been associated with increased cardiovascular morbidity and mortality.[3] Previous studies have suggested that the innate immune system plays a key role in the development of cardiovascular disease in HD patients.[4]

The complement system is a major component of innate immunity and activation of this system induces an inflammatory response.[5] Complement activation can occur via three pathways: the classical pathway (CP), lectin pathway (LP) and alternative pathway (AP). Regardless of the trigger, all pathways lead to the cleavage of C3. In the end, complement activation leads to the generation of C5a, a powerful anaphylatoxin and C5b-9 also known as the membrane attack complex. Initially, the functions of the complement system were thought to be limited to opsonization and elimination of pathogens. However, nowadays this system is known to have numerous functions and complement has been shown to be involved in the pathogenesis of various diseases.[6]

For decades, HD has been known to be associated with complement activation.[7] In dialysis, complement activation is mainly caused by the interaction of blood with the HD membrane.[4] Regardless of the efforts to improve biocompatibility, complement activation still occurs in HD, even with modern membranes.[8–10] It has been hypothesized that complement activation leads to HD-induced inflammation and thereby increases the subsequent cardiovascular risk.[4] In accordance, several studies have shown an association between complement and cardiovascular events (CV-event).[8,11–14] However, the link between complement activation products and CV-events remains poorly characterized.[15] Only *Lines et al.* reported an association in HD patients between soluble C5b-9 and cardiovascular risk. Furthermore, previous experimental studies proposed a link between HD-induced complement activation, pro-inflammatory cytokines, and the coagulation system.[10,16]

We hypothesize that an unfavorable complement profile is seen in HD patients who will develop a CV-event. To investigate the mechanism of increased cardiovascular risk in HD, we measured complement activation, pro-inflammatory cytokines, and a pro-thrombotic factor during one HD session in patients that developed a CV-event during follow-up and compared this to patients without a CV-event during follow-up.

Materials and methods

Study population and design

A cohort of 55 hemodialysis patients from Dialysis Center Groningen and the University Medical Center Groningen were followed for a maximum of 45 months. The original cohort was composed out of 109 patients; however, due to a lack of samples, only 55 could be included in this study. The protocol

has been previously described.[2] In short, patients were included if the duration of HD therapy was longer than 3 months. Patients with severe heart failure (NYHA class IV) were excluded.

Dialysis settings

Patients were on maintenance HD treatment for three times a week with a low-flux polysulfone hollow-fiber dialyzer (F8; Fresenius Care, Bad Homburg, Germany). The dialysate temperature was kept on 36.0 or 36.5°C. The ultrafiltration rate was constantly 500 mL/min and the blood and dialysate flow rate was 250–350. Blood samples were taken just before the start of the session, and after 30, 60, 180 and 240 minutes.

Inflammatory markers

Patient characteristics were extracted from patient records. TNF-α was measured by Quantikine HS Human Immunoassay. IL-6 and IL-10 were determined using a quantitative sandwich enzyme immunoassay technique (R&D System Inc). Von Willebrand Factor was measured by enzyme-linked immunosorbent assay (Dakopatts).

Quantification of complement proteins

C3d was measured by sandwich enzyme immunoassay as previously described.[17] Quantitative antigenic assay for C3 was performed by the radial immunodiffusion technique with monospecific anti-sera.[17] As a measure of complement activation C3d/C3 ratio was determined by dividing the C3d values by the C3 concentration. Additionally, Properdin and MBL concentrations were measured as described earlier.[17,18]

Definition of endpoint

The end-point of the study was defined as the time to the first CV-event. CV-event included cardiac, cerebrovascular or peripheral vascular events. The occurrence of a cardiac event was defined as an ischemic heart disease (unstable angina pectoris, myocardial infarction, Coronary Artery Bypass Grafting (CABG) and/or Percutaneous Coronary Intervention (PCI), sudden cardiac death and congestive heart failure. In order to classify as acute myocardial infarction, we used two out of three criteria: clinical status, elevated heart enzymes, and EKG changes. Cerebrovascular events were defined as stroke, ischemic insult, or newly diagnosed >70% stenosis of the extracranial carotid artery. Strokes and ischemic insults had to be verified by CT or MRI. Peripheral vascular disease was defined as having intermittent claudication with angiographically or sonographically proven stenosis >50% of the major arteries of the lower limbs or ulcers caused by atherosclerotic stenosis or surgery for this disorder. Transplantation was a censoring event and the transplantation date was considered as the final follow-up date.

Statistics

Statistical analysis was performed using IBM SPSS 22.0 (IBM Corporation, Chicago, IL, USA). Normally distributed data are presented as mean ± standard deviation, whereas non-normally distributed data are shown as median with interquartile range [IQR]. Nominal data are displayed as total number

of patients with percentage [n (%)]. Differences between groups were assessed with the student t-test and the paired t-test was used to compare values of a single variable during different time points within the HD session.

Ethics

This study was in accordance with the Declaration of Helsinki and approved by the Medical Ethical Committee.

Results

Patients characteristics

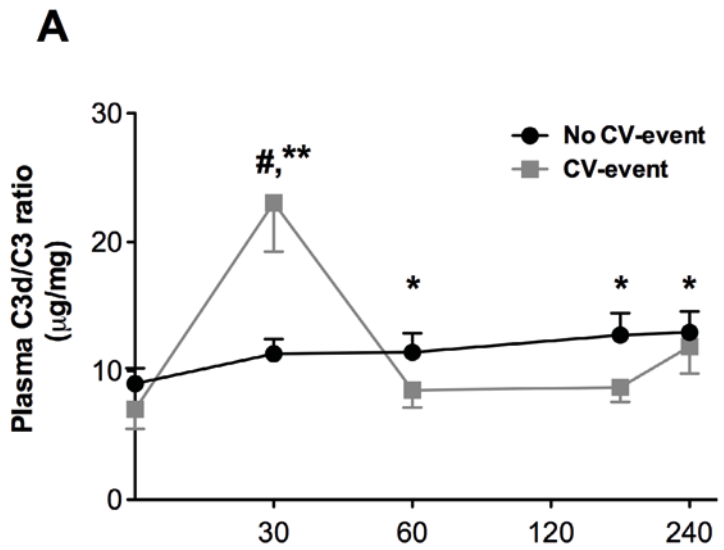
Blood samples from 55 patients on maintenance HD were available, of which 35 were male and 20 female. The mean age was 62 ± 15 years and baseline dialysis vintage was 1.2 years [IQR: 0.6 – 3.9 years]. The median follow-up of the study was 32 months and during this time 17 patients (31%) developed a CV-event, whereas 16 patients died (29%). Among the patients that developed CV-events, 35% had acute coronary syndrome, 17% needed coronary artery bypass surgery, 11% developed congestive heart failure, 17% had a cerebro-vasculair accident and 17% developed peripheral vascular disease. Next, we created two different groups; the patients that developed a CV-event during follow-up (CV-event group) and the patients that remained event-free (event-free group).

Complement activation

To assess complement activation we determined the C3d/C3-ratio of the HD patients during one HD session prior to the follow-up. The C3d/C3-ratio at the start of the HD session was not statistically different between the patients that would develop a CV-event (7.0 ± 6.2) compared to the patients that would not (9.0 ± 7.4). Surprisingly, at the end of the HD session, the C3d/C3-ratio was also not statistically different between the two groups (CV-events: 11.8 ± 8.5, event-free: 12.9 ± 10.0). However, when the intradialytic C3d/C3-ratios were compared between the two groups, clear differences were seen (Figure 1). At 30 minutes intradialysis, there was a significant increase in the C3d/C3-ratio in the CV-event group compared to the patients who remained event free. During these initial 30 minutes, the C3d/C3-ratio increased by 3.29 fold in the CV-event group and by only 1.26 fold in the event-free group ($P<0.01$).

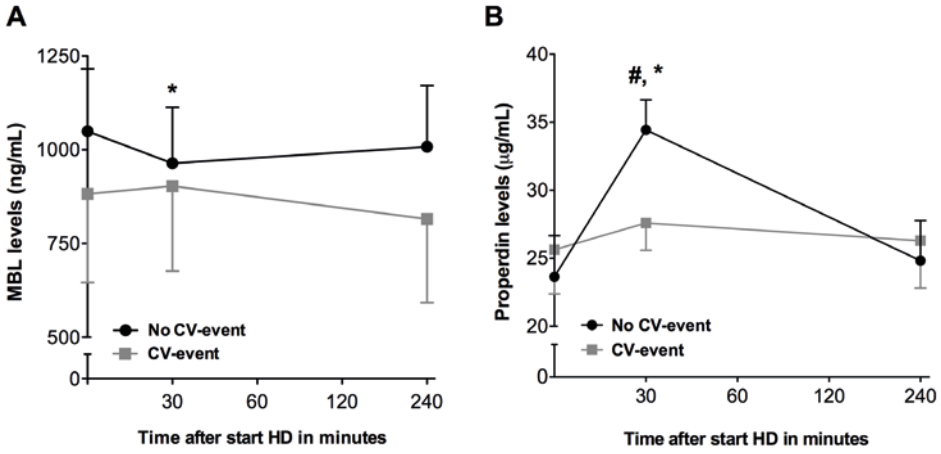
We next set out to assess the contribution of the AP and LP to HD-induced complement activation. Properdin and MBL levels were measured in a subgroup of 30 patients (Figure 2). MBL and properdin levels were comparable between the two groups at the start and end of the HD session. Conversely, at 30 minutes intradialysis MBL levels decreased significantly in the event-free group but not in the CV-event group ($P<0.05$). Furthermore, properdin levels were significantly lower at 30 minutes in the CV-event group, compared to the event-free group. To summarize, MBL consumption was seen in the event-free group implying there is binding of MBL to the HD membrane. In contrast, the lower properdin levels in the CV-event group suggest binding of properdin to the HD membrane.

Figure 1
C3d/C3-ratios during hemodialysis



*Two different groups were created, the patients that developed a cardiovascular event during follow-up (CV-event) and the patients that remained event-free (no CV-event). The data are presented as mean ± SEM and C3d/C3-ratio was calculated by dividing the C3d values (at µg/mL) by the C3 levels (in mg/mL). The C3d/C3-ratio was determined at the start of hemodialysis session and 30, 60, 180 and 240 minutes after. Differences between the two groups were assessed by the student t-test and the paired t-test was used to compare C3d/C3-ratio at different time points within one group (*P<0.05, **P<0.01, ***P<0.001). The hashtag above the bars denotes a significant difference between the two groups, whereas the asterisk above the bars denotes a significant difference compared to baseline within the group. The number of subjects is 17 in the 'CV-event group' and 38 in the 'No CV-event group'.*

Figure 2 Intradialytic levels of properin en MBL



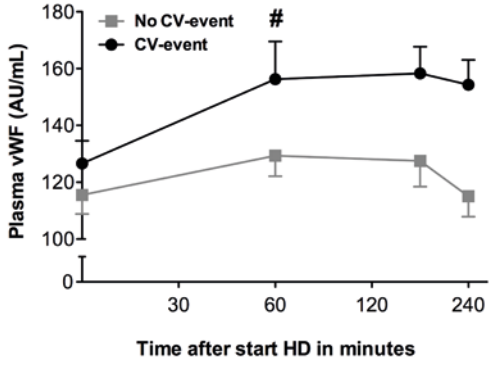
*Two different groups were created, the patients that developed a cardiovascular event during follow-up (CV-event) and the patients that remained event-free (no CV-event). The data are presented as mean ± SEM. (A) The levels of MBL were determined at the start of hemodialysis session and 30 and 240 minutes after. (B) The levels of properdin were determined at the start of hemodialysis session and 30 and 240 minutes after. Differences between the two groups were assessed by the student t-test and the paired t-test was used to compare levels at different time points within one group (*P<0.05, **P<0.01, ***P<0.001). The hashtag above the bars denotes a significant difference between the two groups, whereas the asterisk above the bars denotes a significant difference compared to baseline within the group. The number of subjects is 17 in the 'CV-event group' and 38 in the 'No CV-event group'.*

Inflammatory and pro-thrombotic factors

Finally, we determined cytokines and Von Willebrand factor (vWF) to investigate if complement activation during HD is accompanied by a pro-inflammatory response and a pro-thrombotic state. During HD distinct time-courses were observed between the two groups for levels of vWF (Figure 3). In the CV-event group, vWF levels increased steadily during the session, however, they did not reach statistical significance. Nevertheless, compared to patients without a CV-event, the CV-event group had significantly higher levels of vWF after 180 and 240 minutes ($P<0.05$).

Cytokines such as tumor necrosis factor-α (TNF-α) may initiate inflammation and are therefore believed to play a role in dialysis-related cardiovascular risk. Levels of TNF-α rose significantly during the HD session in both groups (Figure 4A). In the CV-event group, levels peaked at 180 minutes after the start of the HD session ($P<0.01$) and were significantly higher than the event-free group ($P<0.05$). Furthermore, in the event-free group, the maximum TNF-α levels were reached at the end of the session ($P<0.001$).

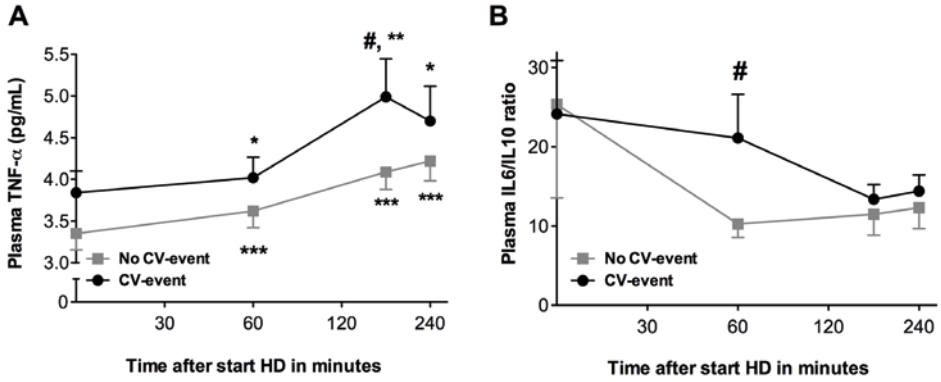
Figure 3
Levels of von Willebrand factor during hemodialysis



*Two different groups were created, the patients that developed a cardiovascular event during follow-up (CV-event) and the patients that remained event-free (no CV-event). The data are presented as mean ± SEM. (A) Von Willebrand factor (vWF) was determined at the start of hemodialysis session and 60, 180 and 240 minutes after the start of the session. Differences between the two groups were assessed by the student t-test and the paired t-test was used to compare C3d/C3-ratio at different time points within one group ((*P<0.05, **P<0.01, ***P<0.001). The hashtag above the bars denotes a significant difference between the two groups, whereas the asterisk above the bars denotes a significant difference compared to baseline within the group. The number of subjects is 17 in the 'CV-event group' and 38 in the 'No CV-event group'.*

To evaluate the relation between anti-inflammatory cytokines and pro-inflammatory cytokines, we determined the IL-6/IL-10 ratio (Figure 4B). Interestingly, IL-6/IL-10 ratios were the highest in both groups at the start of the HD session and a decreasing trend was seen during the session, although not significant. Moreover, at 60 minutes intradialysis an important decrease in the IL-6/IL-10 ratio occurred in the event-free group, indicating a shift towards a less inflammatory profile. However, IL-6/IL-10 ratios remained elevated in the HD patients that will develop a CV-event, revealing a significant difference between the groups at this time point ($P<0.05$). Overall, enhanced levels of pro-inflammatory and pro-thrombotic mediators seem to prelude the development of CV-events in HD patients.

Figure 4
Levels of TNF-α and the IL-6/IL- 10-ratio during hemodialysis



*Two different groups were created, the patients that developed a cardiovascular event during follow-up (CV-event) and the patients that remained event-free (no CV-event). The data are presented as mean ± SEM. (A) The levels of TNF-α were determined at the start of hemodialysis session and 60, 180 and 240 minutes after the start of the session. (B) Levels IL-6 and IL-10 were determined at the start of hemodialysis session and 60, 180 and 240 minutes after. The IL-6/IL- 10 ratio was calculated by dividing the IL-6 (in pg/mL) values by the IL-10 levels (in pg/mL). Differences between the two groups were assessed by the student t-test and the paired t-test was used to compare C3d/C3-ratio at different time points within one group (*P<0.05, **P<0.01, ***P<0.001. The hashtag above the bars denotes a significant difference between the two groups, whereas the asterisk above the bars denotes a significant difference compared to baseline within the group. The number of subjects is 17 in the 'CV-event group' and 38 in the 'No CV-event group'.*

Discussion

Hemodialysis treatment balances between the dangers of advanced uremia and the inherent risks related to this form of RTT.[19,20] The higher cardiovascular risk seen in this population is not only related to ESRD but it is also associated with the HD procedure itself.[2] Innate immunity has been proposed to be the missing link in the mechanism of CV-events in HD patients.[4] We observed distinct differences in molecular profiles during HD of patients that will later develop CV-events compared to those who remained event-free during follow-up. At the start of dialysis, a unique peak in complement activation was only seen in patients of the CV-event group. Furthermore, enhanced inflammation and coagulation accompanied the complement activation seen in HD patient that will develop CV-events. Altogether these three elements showed different dynamics, with complement activation possibly initiating these processes. Moreover, these processes arose long before the actual development of the CV-event.

Despite significant advances in the biocompatibility of HD membranes, complement activation remains an undesired but relevant issue.[8,15] Higher levels of complement components, as well as loss of complement inhibitors, have been associated with a higher risk for cardiovascular disease in HD patients.[8,11–14] Recently it was reported that complement activation prior to an HD session was associated with the occurrence of CV-events in HD patients.[15] Here, we showed that patients that will develop a CV-event exhibit a intradialytic peak in C3 activation, possibility suggesting that intradialytic complement activation results in CV-events. Our study is the first, to our knowledge, to assess the relationship between intradialytic complement activation and subsequent outcome. In accordance, previous studies have shown that activation of the complement system peaks during the first 15 to 30

minutes of the HD session.[21] However, the mechanism by which complement activation increases the risk for cardiovascular disease remains largely unknown.

The LP and AP initiate complement activation during HD.[22,23] In our study, we only found MBL consumption in the event-free group, implying that this decrease is actually beneficial. In accordance, MBL has been proposed to be involved in the removal of atherogenic particles, thereby decreasing atherosclerosis. Our previous data showed that higher MBL levels in HD patients were associated with protection against cardiovascular disease.[9] We also found a rise in properdin levels in the event-free group. Properdin, unlike other complement factors, is produced by leukocytes, predominately neutrophils.[24] Therefore, the increase in properdin is presumably the result of leukocyte activation by the HD membrane leading to degranulation.[25] Since, this rise was not seen in the CV-event group, we speculate that this was due to properdin consumption by AP activation in these patients.

We found higher TNF-a levels and IL-6/IL-10 ratios in patients that would develop a CV-event. TNF-α and IL-6 are potent cytokines that can initiate a powerful pro-inflammatory reaction.[26,27] If this response is not contained, it can lead to hypotension, organ dysfunction, and eventually result in death. Elevated levels of these cytokines have also been related to an increased risk for CV-events in the general population and in HD patient.[28–31] In contrast, IL-10 is a major anti-inflammatory cytokine with the ability to suppress the production and secretion of pro-inflammatory mediators in leukocytes, thereby effectively controlling the inflammation.[32] The IL-6/IL-10 ratio has previously been linked to outcome after inflammatory disorders and to the development of HD-induced left ventricular dysfunction.[33–35] In ex-vivo models, the induction of IL-6 during the bio-incompatibility reaction was shown to be completely complement-dependent, while the induction of TNF-α was only partially complement-dependent.[36] In addition, in a primate model of HD, complement inhibition leads to enhanced levels of IL-10, demonstrating the relationship between the two systems.[37]

Thrombosis is a key element in the development of cardiovascular disease. Previously, *Péquériaux et al.* reported that vWF is a good predictor of CV-events in patients undergoing RRT.[38] Von Willebrand factor is a glycoprotein involved in hemostasis but vWF is also a marker of endothelial cell activation.[39] We found significantly higher levels of vWF in the group of patents who developed CV-events, which could be evidence of a prothrombotic state. The link between the complement system and thrombosis is not new in HD.[40] Complement receptors on leukocytes are important for the formation of platelet-leukocytes complexes, which contributes to thrombotic processes.[41] In addition, complement activation during HD induces the production of pro-coagulation factors.[42] Moreover, plasma levels of C3 correlated with a denser clot structure in HD patients.[43]

There is a growing body of data supporting a role for the complement system in the development of cardiovascular disease. *Ekdahl et al.* proposed that complement activation initiates an inflammatory cascade and amplifies pro-thrombic processes.[4] For the first time, to our knowledge, we demonstrated intradialytic differences in complement activation, inflammation and a pro-thrombotic factor in HD patients that will develop a CV-event compared to HD patients that will not. Future studies have to determine whether these three processes are collinear or parallel in the mechanism of CV-events in HD patients.

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